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VOLATILE ORGANIC COMPOUNDS AND MALIGNANT PLEURAL MESOTHELIOMA: SIMPLY BREATHTAKING?

Kevin LAMOTE

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Promotor: Prof. dr. Jan P. van Meerbeeck

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Promotor

Prof. dr. Jan P. van Meerbeeck

Department of Internal Medicine, Ghent University, Faculty of Medicine and Health Sciences, Ghent, Belgium.
Department of Thoracic Oncology/MOCA, Antwerp University Hospital, Edegem, Belgium.

Members of the examination committee

Prof. dr. Joris R. Delanghe

Chairman
Department of Clinical Biology, Microbiology and Immunology, Ghent University Hospital, Faculty of medicine and Health Sciences, Ghent, Belgium.

Prof. dr. Frederik-Jan van Schooten

Department of Pharmacology & Toxicology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, The Netherlands.

Prof. dr. Arnaud Scherpereel

Department of Pulmonary and Thoracic Oncology, CHRU de Lille and University of Lille II, Lille, France.

Prof. dr. Evelien Smits

Center for Oncological Research (CORE), University of Antwerp, Faculty of Medicine and Health Sciences, Antwerp, Belgium.
Laboratory of Experimental Hematology, Tumor Immunology Group (TIGR), Vaccine and Infectious Disease Institute (VAXINFECTIO), University of Antwerp, Faculty of Medicine and Health Sciences, Antwerp, Belgium.
Center for Cell Therapy and Regenerative Medicine, Antwerp University Hospital, Edegem, Belgium.

Prof. dr. Lutgart Braeckman

Department of Public Health, Ghent University Hospital, Faculty of Medicine and Health sciences, Ghent, Belgium.

Prof. dr. Veerle Surmont

Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium.
Department of Internal Medicine, Ghent University, Faculty of Medicine and Health sciences, Ghent, Belgium.

Prof. dr. Eric Derom

Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium.
Department of Internal Medicine, Ghent University, Faculty of Medicine and Health sciences, Ghent, Belgium.

<<Be faithful in small things, because it is in them that your strength lies.>>

To Ann-Sophie

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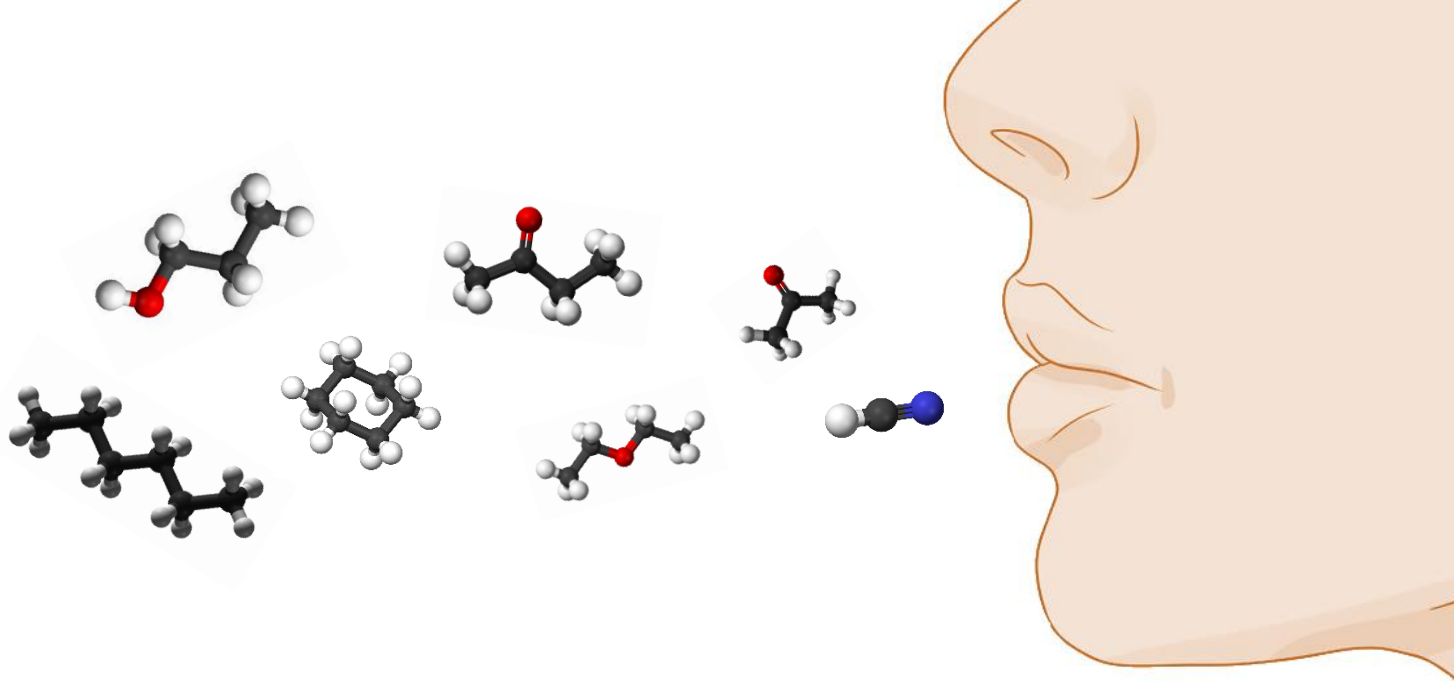
ABBREVIATION LIST

AEx	Asymptomatic persons with historical occupational Asbestos Exposure
ANOVA	Analysis of Variance
ARD	Patients with benign Asbestos-Related Diseases
AUC_{ROC}	Area Under the Receiver Operator Characteristic Curve
BAP1	BRCA-Associated Protein 1
BLD	Patients with Benign, non-asbestos-related Respiratory Diseases
CA125	Cancer Antigen 125
CCL2	C-C Motif Chemokine Ligand 2
CDKN2A	Cyclin-dependent Kinase Inhibitor 2A
CK	Cytokeratin
CO_2	Carbon Dioxide
CT	Computed Tomography
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DPT	Diffuse Pleural Thickening
EBC	Exhaled Breath Condensate
EGFR	Epidermal Growth Factor Receptor
eNose	Electronic Nose
EORTC	European Organisation for Research and Treatment of Cancer
e-P/D	Extended Pleurectomy/Decortication
EPP	Extrapleural Pneumonectomy
FDA	Food and Drug Administration
FeNO	Fraction of Exhaled Nitric Oxide
^{18}F-FDG	2-deoxy-2-[fluorine-18]fluoro-D-glucose
GC-MS	Gas Chromatography – Mass Spectrometry
GPI	Glycosylphosphatidylinositol
HC	Healthy Control subject without occupational asbestos exposure
HMGB1	High Mobility Group Box 1

HRCT	High Resolution Computed Tomography
IABR	International Association for Breath Research
IASLC	International Association for the Study of Lung Cancer
IGF	Insulin Growth Factor
IMS	Ion Mobility Spectrometry
iNOS	Inducible form of Nitric Oxide Synthase
kDa	Kilodalton
kPa	Kilopascal
LASSO	Least Absolute Shrinkage and Selection Operator
LC	Lung Cancer patient
LDCT	Low Dose Computed Tomography
LOD	Limit of Detection
LOOCV	Leave-One-Out Cross-Validation
LOQ	Limit of Quantification
LT	Leukotriene
MCC/IMS	Multicapillary Column/Ion Mobility Spectrometry
MPM	Malignant Pleural Mesothelioma
MPF	Megakaryocyte Potentiating Factor
MRI	Magnetic Resonance Imaging
m/z	Mass-to-Charge Ratio
NF2	Neurofibromin 2 (Merlin)
NF-κB	Nuclear Factor-κB
NIH	National Institutes of Health
NIST	National Institute of Standards and Technology
NO	Nitric Oxide
NPV	Negative Predictive Value
NSCLC	Non-small cell lung cancer
OPN	Osteopontin
OS	Overall Survival
PD-1	Programmed Death 1 Receptor
PD-L1	Programmed Death 1 Ligand
PET	Positron Emission Tomography

PFS	Progression Free Survival
PMA	Pre-Market Approval
PORT	Postoperative Radiotherapy
PP	Pleural Plaques
PPV	Positive Predictive Value
PTFE	Polytetrafluoroethylene
RAGE	Receptor for Advanced Glycation End Products
RAS	Rat Sarcoma
Rb	Retinoblastoma protein
RNS	Reactive Nitrogen Species
ROC	Receiver Operator Characteristic Curve
RON	Receptor d'Origin Nantais
ROS	Reactive Oxygen Species
RT	Retention Time
SMRP	Serum Mesothelin-related Protein
TIL	Tumour-infiltrating Lymphocytes
TNF	Tumour Necrosis Factor
TRX	Thioredoxin-1
US	United States
VATS	Video-Assisted Thoracoscopic Surgery
VEGF	Vascular Endothelial Growth Factor
VOC	Volatile Organic Compound
WHO	World Health Organisation
WNT	Wingless-related Integration Site
WT1	Wilms Tumour Protein 1



PART I:

GENERAL INTRODUCTION

CHAPTER 1
ASBESTOS

1.1 Asbestos

Asbestos are a group of naturally occurring, inert, hydrated silicate minerals. These fibres are interesting because of their important properties for industrial use, such as a high tensile strength, and resistance to thermal, chemical, and electrical degradation. Asbestos is derived from the Greek '$\alpha\sigma\beta\varepsilon\sigma\tau\sigma\varsigma$' and means 'inextinguishable'. Dependent on their characteristics, two classes of asbestos fibres can be distinguished: serpentines and amphiboles (Figure 1, Table 1) [1, 2]. The only serpentine asbestos is chrysotile or white asbestos. Chrysotile asbestos consists of a sheet of nanosized tubular fibres with a hollow core and represents over 95% of all asbestos produced and consumed [3]. These fibres are curlier and more bendable and are more prone to dissolve and be cleared in tissues. Furthermore, there are five types of amphibole asbestos fibres: two commercially used, i.e. crocidolite (blue asbestos or riebeckite) and amosite (brown asbestos or grunerite) and three non-commercially used, i.e. actinolite (green asbestos), anthophillite (yellow asbestos), and tremolite (grey asbestos). The amphibole fibres are known to be straight and sharp and more resistant to chemical and biological dissolution resulting in a greater durability and biopersistence of these fibres.

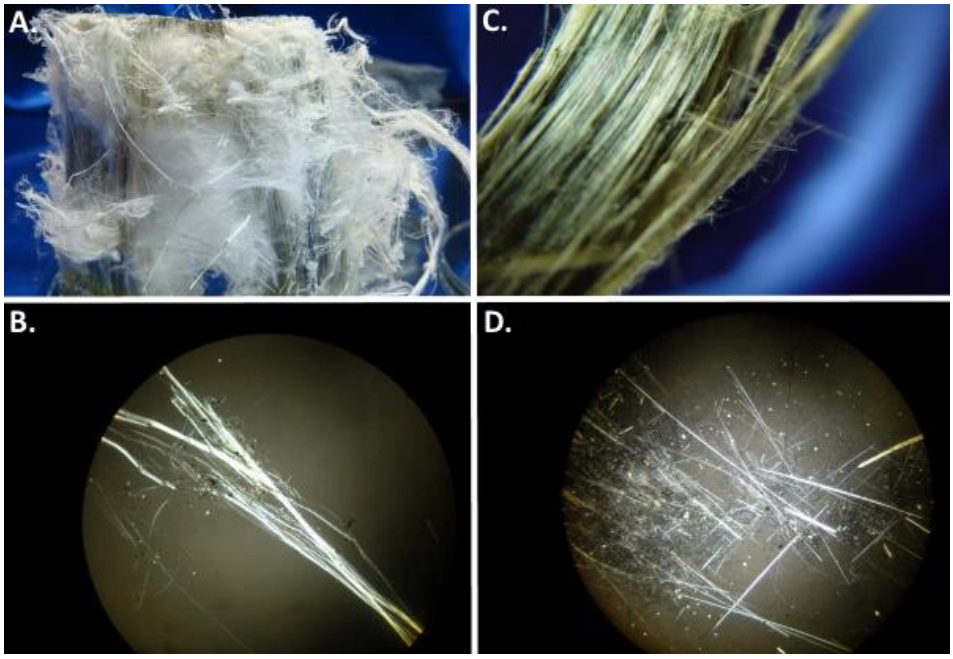


Figure 1: General and microscopic representation of a serpentine (chrysotile, **A and B**) and an amphibole (amosite, **C and D**).

Table 1: Asbestos classes and properties

Class	Properties	Name
Serpentine	Curly, bendable, short biopersistance	Chrysotile (white asbestos)*
Amphibole	Straight, rigid, needle-like, strong with a long biopersistance	Actinolite (green asbestos) Amosite (brown asbestos)* Anthophillite (yellow asbestos) Crocidolite (blue asbestos)* Tremolite (grey asbestos)

*Commercially used

Although chrysotile fibres are thought to be less carcinogenic, all asbestos fibres have been shown to be carcinogenic and are classified a group 1 human carcinogen by the International Agency for Research on Cancer (IARC) of the World Health Organisation (WHO) [4, 5].

1.2 Past and present consumption

Asbestos fibres are known to exist for more than 4000 years. The Egyptians used them for embalming mummies and in the Roman times, asbestos fibres were used as insulating material and as wicks of lamps and candles. Furthermore, amphibole fibres occur naturally as contaminant in the minerals in rock formations [6]. Because of their interesting technical properties, the fibres have been used as insulation material in factories, schools, homes and for ship building. Besides, it was also used to make automobile brakes and clutch parts, roofing shingles, ceiling and floor tiles, and woven into textiles for fire and heat protection [6]. Industrial production of asbestos began in the late 19th century, but boomed by the World Wars. The first cases of asbestos-associated fibrosis were described in the early 1900s but the association between lung carcinoma and asbestos fibres was established half a century later by the mid-1950s [7], whereas the association between asbestos and malignant pleural mesothelioma (MPM) was recognized in the 1960 by Wagner who initiated a case-control study in South-African miners [8]. In the Netherlands, the first cases of mesothelioma were seen in the beginning of the 1970s [9, 10]. Despite these known health effects, asbestos use continued and peaked in the ‘70s [2]. Today, still more than 2 million tons is annually produced worldwide with Russia being the leading producer, followed by China, Kazakhstan, Brazil, Zimbabwe and Columbia, together accounting for 96% of the world production of asbestos [11]. Very recently, Canada was included in this list. But since April 2016, it has committed to

move forward with a plan to ban the use of asbestos and as a first step, has banned the export and use of asbestos in new constructions since then.

Asbestos use is now banned in 55 countries, including all members of the European Union [12]. In developing countries in need for industrial growth, where too often little or no protection of workers and communities exists, the asbestos cancer pandemic may be the most devastating. A shift in asbestos consumption is seen to Asia, with China being the largest consumer of asbestos in the world, followed by India, Russia, Kazakhstan, Thailand, Ukraine, and Uzbekistan. Belgium was one of the highest asbestos consumers in the past, which is reflected in the high standardized mortality for mesothelioma and asbestosis in 2004 (Figure 2) [13]. In Belgium, the use, production or marketing of asbestos fibres is completely prohibited since 1998.

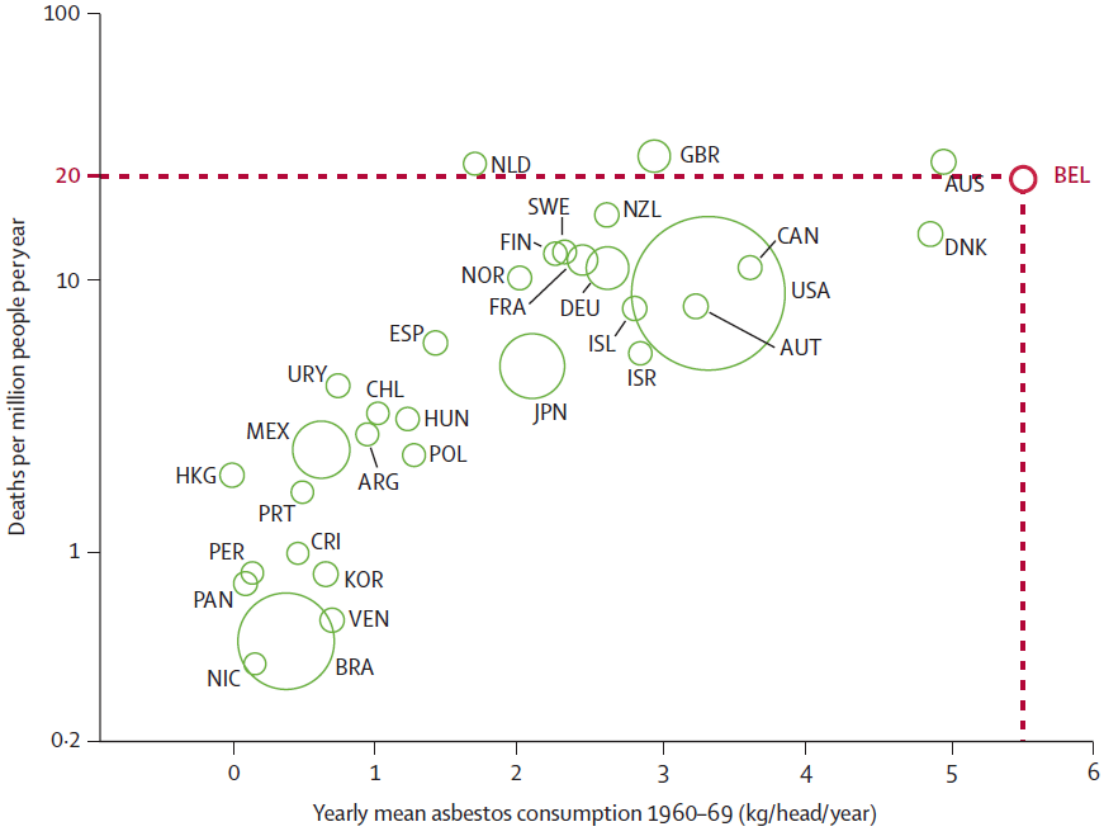


Figure 2: Association between historical asbestos consumption and mortality from mesothelioma and asbestosis in men [13].

CHAPTER 2
ASBESTOS-RELATED DISEASES

2.1 Introduction

After inhalation of asbestos fibres, the fibres can act on the lung parenchyma (bronchioles, bronchi and lung interstitium) and on the pleura [14, 15]. The latter is a serous membrane covering the lungs and chest cavity, and exists of a monolayer of mesothelial cells. The part covering the lungs is the visceral pleura and also covers the interlobar fissures. The other part, the parietal pleura, covers the rest of the thoracic cavity and the diaphragm. In between the two pleural blades, a small virtual space is present (the pleural cavity) which is filled with 0.1-0.2 ml/kg body weight of pleural fluid (Figure 3).

Inhalation of asbestos fibres can cause benign (pleural plaques, asbestosis, diffuse pleural thickening, pleural effusions) or malignant (lung cancer, malignant mesothelioma) respiratory diseases [16]. This chapter discusses both types of asbestos-related diseases.

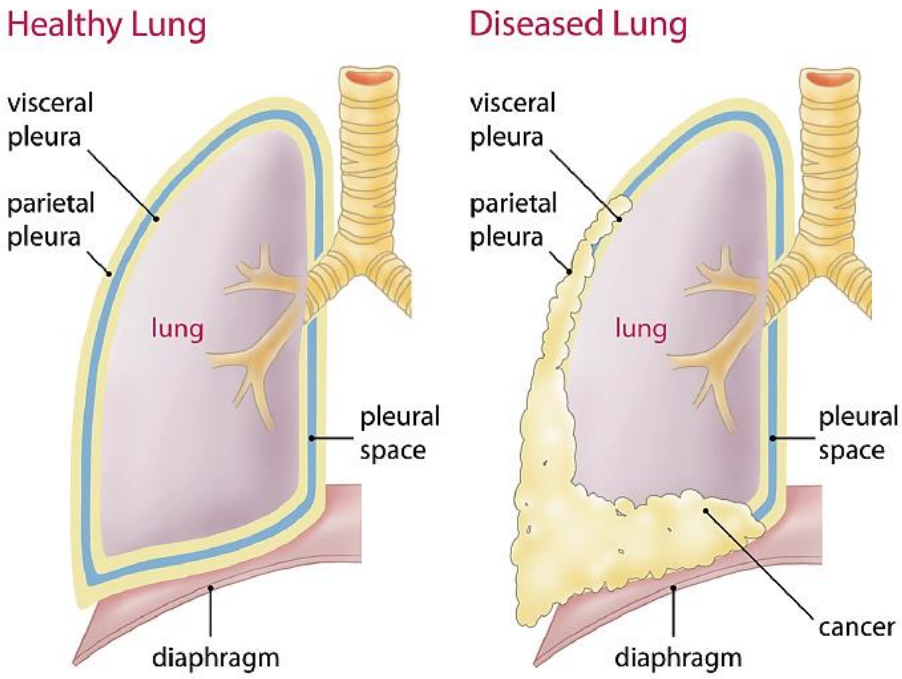


Figure 3: Normal **(left)** and diseased **(right)** pleura [17].

2.2 Benign diseases

2.2.1 Pleural plaques

Pleural plaques (PP) are the most common manifestation of asbestos exposure [18]. PPs are small areas of hyaline fibrosis which can become calcified and mostly originate on the parietal pleura of the chest wall and diaphragm [19]. Because of the layers of hyalinised collagen fibres, plaques appear white and are often multiple and bilateral [20, 21]. The plaques occur with lower inhaled asbestos burdens and can result from small temporally exposures. Plaques occur 20-30 years after asbestos exposure in approximately 50-60% of individuals with a heavy or prolonged asbestos exposure [18, 20]. Pleural plaques can be seen on chest X-ray, although a computed tomography (CT) is more sensitive. Pleural plaques are asymptomatic and do not undergo malignant transformation nor does their presence increases the risk for MPM [22]. Hence, PPs serve as a marker of asbestos-exposure.

2.2.2 Asbestosis

Asbestosis is a bilateral, diffuse interstitial fibrosis of the lung parenchyma caused by inhalation of asbestos fibres [19]. It is linked to high cumulative doses of asbestos exposure, with the presence of asbestos bodies in the alveoli [21]. There is a latency period of 20 years between exposure and onset of symptoms such as breathlessness and a reduced exercise tolerance. In contrast to the other benign asbestos-related diseases, asbestosis can be a fatal due to its evolution to respiratory failure [23].

2.2.3 Diffuse pleural thickening

Diffuse pleural thickening (DPT) is essentially a plaque that extends over a wide range of the visceral pleura with fusion to the parietal pleura. DPT can result from multiple benign asbestos-related pleural effusions, but is not a pathognomic marker for asbestos exposure [19, 20]. In contrast to plaques, it rarely calcifies, is irregularly shaped and induces fusion of the pleural layers which can result in functional impairment. It occurs less frequent than pleural plaques and is rarely bilateral [20]. In unique cases, DPT can cause rounded atelectasis

or folded lung [24], in which the fibrous tissue matures, contracts and causes the fissural pleura to hunch into the lung. Patients with rounded atelectasis are asymptomatic in most cases, but can present with dyspnoea if the atelectatic volume is large and lung function is compromised. An important differential diagnosis is to be made with lung cancer.

2.2.4 Pleural effusions

These are the earliest abnormality seen after asbestos exposure, usually between 10-20 years after exposure [19, 20]. Effusions do not contain asbestos fibres and tend to be exudative, but have a highly variable composition [25, 26]. Although most effusions are small to moderate, they can occur bilaterally in 10% of cases and effusions typically must be large enough to cause symptoms. When symptomatic, a pleural effusion can manifest with fever, cough, pain and dyspnoea [20]. A benign asbestos-related pleural effusion presents itself without comorbidities or a malignancy within three years. Similar to a pleural plaque, it has no prognostic implications for mesothelioma development, but is only a marker of past asbestos exposure [27]. A pleural effusion does not require specific treatment, except thoracentesis in symptomatic patients. Usually effusions tend to diminish slowly and spontaneously within 1-17 months. However, recurrences are frequent (30-40%) and occur within 3 years. As mentioned earlier, pleural effusions can lead to DPT.

2.3 Malignant diseases

2.3.1 Lung cancer

Although smoking is the main causal agent for lung cancer, asbestos exposure alone increases lung cancer mortality among non-smokers and has a synergistic effect with smoking on lung cancer pathogenesis, rather than additive [28]. In patients with asbestosis, asbestos fibres are often considered the main cause of lung cancer and the presence of asbestosis further increases the lung cancer risk [29]. Even without asbestosis, heavy exposure to asbestos fibres can cause lung cancer. However, in an individual case, the attributable risk of asbestos exposure is not easily separated from the risk from smoking. In a Dutch study, it was estimated that 11.6% of the lung cancer cases that occurred in men aged 55–73 years between 1986 and

1990 were related to asbestos exposure [30]. For lung cancer patients exposed to both smoking and asbestos, an estimated 26% (95% CI 14–38%) of lung cancer deaths were attributable to the interaction between asbestos and smoking [31]. In these lung cancer patients, there were more deaths attributable to smoking only (68%) than asbestos exposure only (2%) [31].

2.3.2 Malignant Mesothelioma

Mesothelioma is a lethal disease related to asbestos-exposure. It can occur on the serosal surfaces of the pleura, peritoneum, pericard and tunica vaginalis [32]. The most common form is malignant pleural mesothelioma (MPM) accounting for approximately 80% of mesotheliomas and originating from the lower parietal pleura and the costadiaphragmatic sinus [32]. It encapsulates the lung resulting in a rind of tumour that covers the lung with minimal penetration of the lung parenchyma. With a mean latency period of 35-40 years after exposure [33, 34], it occurs in a mainly elderly population.

There are typically four major different histological subtypes of mesothelioma: epithelioid, sarcomatoid, desmoplastic and mixed or biphasic (Figure 4) [35].

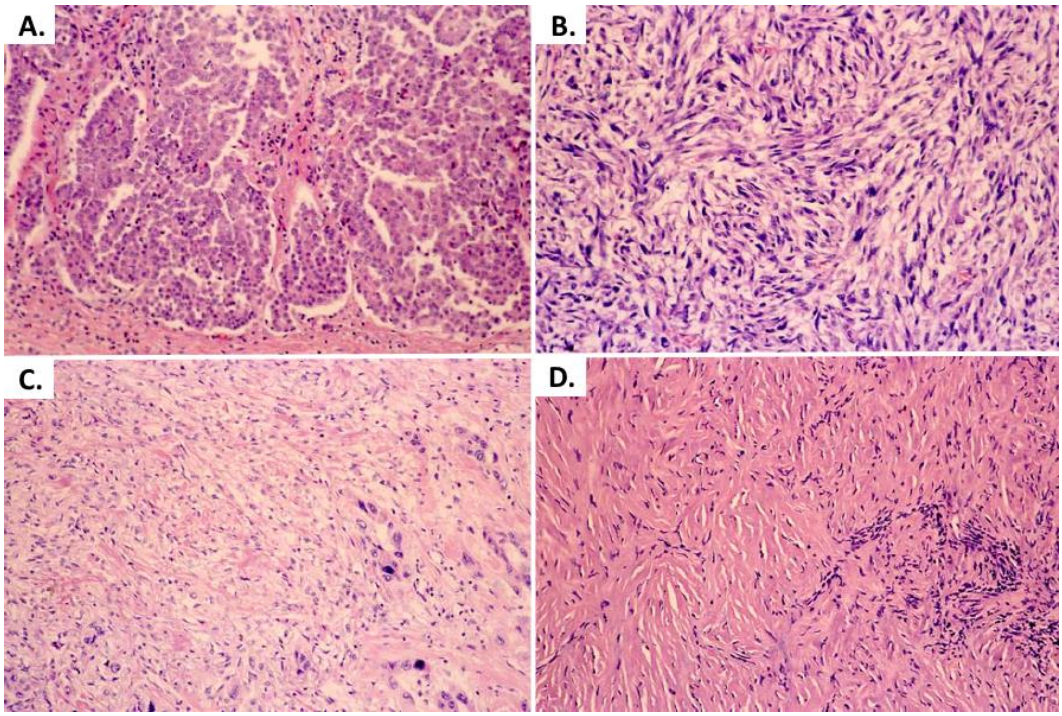


Figure 4: Histology of **A:** epithelioid, **B:** sarcomatoid, **C:** biphasic/mixed, and **D:** desmoplastic mesothelioma [35].

Epithelioid mesothelioma is the most common subtype and consists of polygonal, oval or cuboidal cells that often mimic non-neoplastic reactive mesothelial cells, and is present in approximately 50-60% of mesothelioma patients [20]. It often presents with a pleural effusion. Epithelioid mesothelioma show a wide range of morphologic patterns. The most frequent patterns are (tubulo)papillary, trabecular, adenomatoid (microglandular) and sheet like. Less frequent patterns are small cell, clear cell, pleiomorphic and deciduoid [36, 37]. Recognition of these patterns have no clear prognostic significance. Therefore, the international mesothelioma WHO pathology panel has stated that epithelioid and sarcomatoid mesothelioma be diagnosed without further subclassifiers [37].

Sarcomatoid mesothelioma comprises 10-20% of mesothelioma cases [38] and consists of malignant spindle cells, but can mimic malignant mesenchymal tumours (e.g. fibrosarcoma or leiomyosarcomas). Biphasic or mixed mesothelioma has both epithelioid and sarcomatoid features present in at least 10% of the sample and accounts for 20-35% of mesothelioma cases [36, 37]. A rare variant of sarcomatoid mesothelioma is the highly aggressive desmoplastic subtype. Patients with the epithelioid subtype have better outcomes than those with mixed or sarcomatoid histology.

Furthermore, since the pleura is also a common site for metastatic disease and reactive changes in the pleura, these must not be confused with MPM.

CHAPTER 3
MALIGNANT PLEURAL MESOTHELIOMA

Malignant pleural mesothelioma (MPM) is a highly aggressive cancer originating from the mesothelial cells from the pleural blades surrounding the lungs [14]. By light microscopy, we can differentiate 3 main histological types: epithelioid, sarcomatoid and a mixed form. MPM typically originates from the lower parietal pleura in the costodiaphragmatic sinus. Presenting clinical symptoms are most often dyspnoea and chest pain, each present in 60% of patients [15]. The global attributable proportion of MPM to asbestos exposure is >80% in males [33] and a dose-dependency has been shown. However, no safe threshold of asbestos exposure has been identified below which there is no increased risk for mesothelioma [6]. Other risk factors and cofactors for MPM include naturally occurring fibres (erionite), exposure to ionising radiation, Simian Virus (SV) 40, and a familial predisposition due to a mutation in the *'BRCA Associated Protein 1'* (BAP1) gene [39-47].

3.1 Oncogenesis

The following are factors that determine the risk for MPM according to the asbestos fibre characteristics [48]: the type, biopersistence, dimensions and surface properties of the fibre, the time since first exposure and the cumulative exposure. Depending on the length-to-width ratio and the shape of the fibres, fibres can penetrate to the alveolar regions. There, the fibres are cleared by phagocytosis through alveolar macrophages [49]. Since chrysotile fibres are more prone to dissolve, these are more easily cleared and are considered less carcinogenic than amphiboles. Amphiboles are not prone to acid dissolution, resulting in a slower dissolution kinetic and hence, a longer presence in the body. Furthermore, asbestos fibres produce reactive oxidants by two known mechanisms. The first mechanism involves the iron content of the fibres, also known as acellular or direct oxidant formation (Figure 5) [50]. Asbestos fibres can be clad with up to 30% iron [49, 51] or, in the case of chrysotile, iron can be present as a contaminant of the attached proteins [51, 52]. Ferrous iron (Fe^{2+}) can dissolve from asbestos fibres, inducing oxidative injury with the formation of reactive oxygen species (ROS), such as hydroxyl ($HO^{\bullet}$) and superoxide ($O_2^{\bullet -}$) radicals, from hydrogen peroxide (H_2O_2)

[53] through Fenton chemistry and the Haber-Weiss cycle. The hydroxyl radical is extremely reactive (reaction rate constant $>10^8$ $M^{-1}s^{-1}$), immediately attacking biomolecules in its direct environment. These oxidants induce deoxynucleic acid (DNA) damage either by hydrogen abstraction or addition of $HO^{\bullet}$ to the DNA [51] and oxidation of membrane lipids [54]. Furthermore, the asbestos fibres can physically interfere with the mitotic spindle and induce structural abnormalities and aneuploidy of the mesothelial cells.

The second mechanism of asbestos-induced oxidant formation involves the activation of inflammatory cells like the alveolar macrophages and neutrophils, known as cellular or indirect oxidant formation (Figure 5) [22, 51, 53, 55]. Because asbestos fibres can be long, the fibres cannot be fully engulfed by the macrophages, inducing frustrated phagocytosis. This relentlessly stimulates the alveolar macrophages in generating O_2^-, H_2O_2 and $HO^{\bullet}$, sustaining a constant generation of oxygen species at the asbestos fibre and subsequent DNA damage in mesothelial cells [49-51, 56, 57].

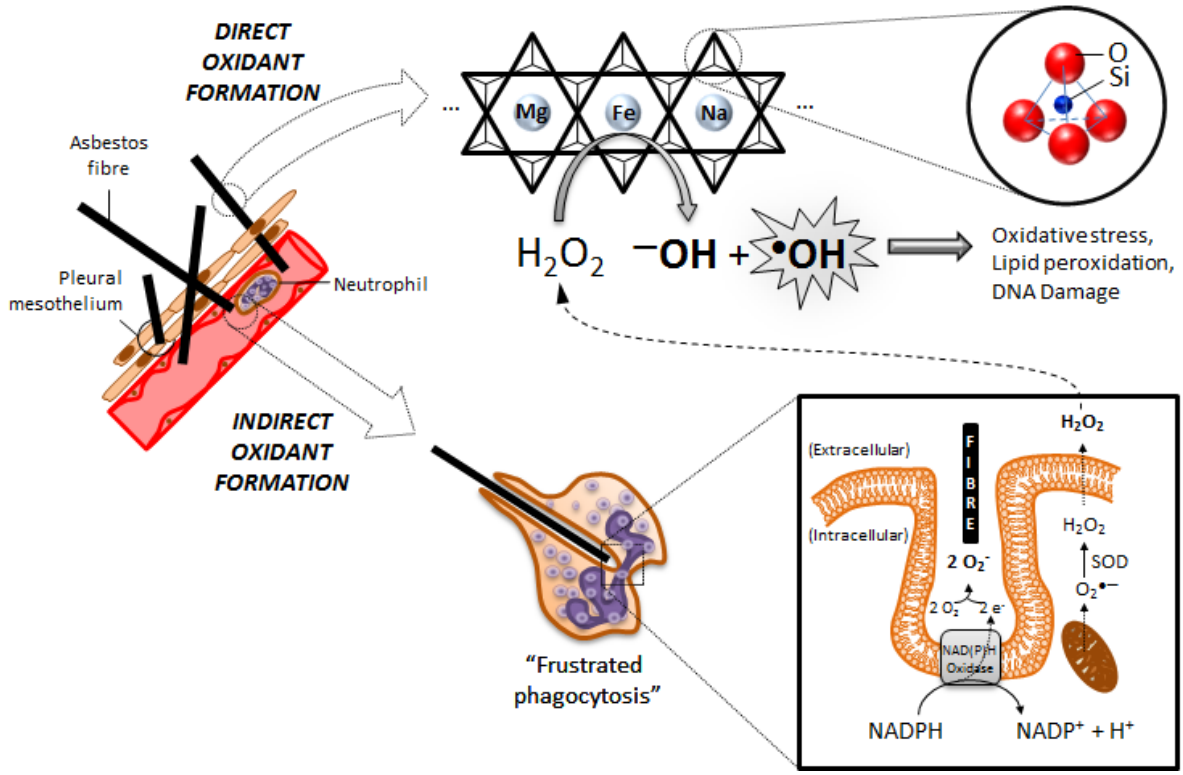


Figure 5: Mechanisms of asbestos-induced oncogenesis. *(Figure by Kevin Lamote)*

Furthermore, asbestos fibres cause mesothelial cell necrosis instead of apoptosis, translocating the nuclear protein high mobility group box 1 (HMGB1) to the extracellular space (Figure 6). HMGB1 stimulates the *"receptor for advanced glycation end products"* (RAGE) on alveolar macrophages, triggering the Nalp3 inflammasome and subsequent IL-1β and tumour necrosis factor (TNF)-α secretion [55, 58]. TNF-α binds its receptor on the mesothelial cells, activating the nuclear factor (NF)-κB pathway and survival of the damaged mesothelial cells. TNF-α protects the cells from asbestos-induced cell death and results in a chronic inflammatory process, supporting the carcinogenic development [55, 59, 60].

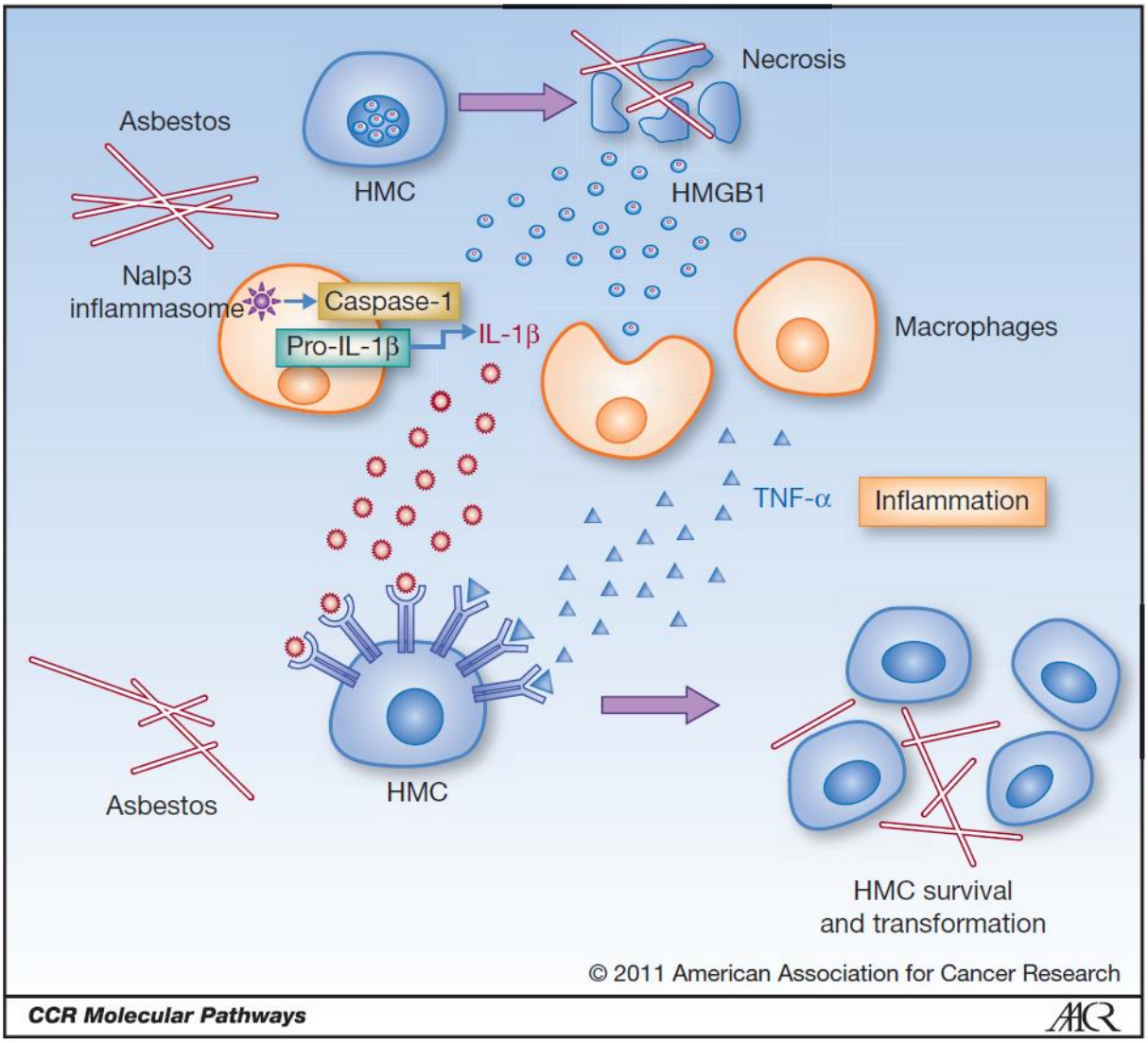


Figure 6: Mesothelioma carcinogenesis [58]. *Asbestos fibres cause necrosis of the mesothelial cells, leading to the release of HMGB1. This causes macrophage accumulation and the secretion of TNF-α. When bound to its receptor on the mesothelial cells, the NF-κB pathway is triggered, inducing cell survival and allowing the mesothelial cells with asbestos-induced DNA damage to survive rather than to die. Together with genetic alterations, mesothelioma can be developed.* HMC: human mesothelial cell. HMGB1: high mobility group box 1. TNF-α: tumour necrosis factor-α.

A major pathway triggered by asbestos fibres is the mitogen-activated protein kinase (MAPK) cascade [61-64]. Several molecular defects have been described in malignant mesothelioma cells, including the cyclin-dependent kinase inhibitor 2A (*CDKN2A)*, neurofibromin 2 (*NF2)* and BRCA-associated protein 1 (*BAP1)* gene, aberrant activation of the Wnt pathway and up-regulation of several receptors including epidermal growth factor receptors (EGFR), insulin-like growth factor (IGF)-1 receptor, vascular endothelial growth factor receptor (VEGFR)-2, RON (Receptor d'Origine Nantais) and c-Met [63, 65-70]. CDKN2A mutations are found in 55% of MPM gene mutations and is the most aberrant mutated gene in MPM [71]. It encodes p14ARF, a proteins that interacts with Mdm2, a negative regulator of p53 [72]. In mesothelioma, extensive gene copy number alterations have been reported with common regions of allele loss being 1p, 3p21, 6q, 9p21, 15q11-15 and 22q [59, 73]. On the other hand, mutations present at high frequencies on other tumours, such as the p53, rat sarcoma (Ras) and retinoblastoma (Rb) proteins, are very rare in MPM. Compared to other tumours like melanoma and non-small cell lung cancer (NSCLC), the mutational load of mesothelioma is lower, making it a less evident target for checkpoint inhibition therapy.

3.2 Epidemiology

Over the last decades, a shift has been observed in the exposure history of mesothelioma cases, from primary and secondary exposure of asbestos workers (e.g. miners handling raw asbestos material) over shipyard or factory workers to end-users often exposed when removing or handling asbestos materials that are still ubiquitously in place, e.g. construction workers, electricians, plumbers, carpenters, mechanics and heating workers with an occupational asbestos-exposure (tertiary exposure) [33]. Even if the occupations with the highest risk of mesothelioma belong to the first group, the number of subjects at risk of MPM is presently much larger in the latter group.

Secondly, there can be para-occupational asbestos exposure in households of asbestos workers due to domestic exposure via work clothes.

Lastly, a certain background exposure exists since asbestos or asbestos-like minerals (erionite) exists naturally in certain areas of the world as a geological component of the soil (Turkey, Corsica, Cyprus and New Caledonia), or can be present in construction materials that contain

asbestos, or occur in the neighbourhood for people living close to asbestos mines or factories [74, 75]. This is called the environmental asbestos exposure.
A dose–effect relationship has been demonstrated with mesothelioma and lung cancer, but it is impossible to define a safe threshold of cumulative exposure below which there is no increased risk [6]. Therefore, all individuals who have been exposed to asbestos, even briefly but intensively, are considered a population at risk for mesothelioma.

3.2.1 Incidence and mortality

The mean latency period between MPM diagnosis and asbestos exposure ranges between 15-67 years with a mean period of 40 years [14]. Due to this long latency period, MPM patients are mostly elderly with a median age at diagnosis in Western countries of 69 years [14]. There are geographical differences in MPM incidence worldwide, ranging from 7 cases per million inhabitants in Japan to 40 cases per million inhabitants in Australia [15, 34]. For Europe, the incidence is reported to be approximately 20 per million inhabitants with large intercountry differences [33]. Belgium had one of the highest historical asbestos consumption worldwide, which is reflected in the high incidence rate of MPM of 39 per million male inhabitants. Since the asbestos industry predominantly occupied males, there is also a gender difference in incidence with 10-66 cases per million in males and 1-2.5 cases per million in females, respectively. Furthermore, given the long latency period and the rather recent bans on asbestos use, the incidence of MPM is expected to increase [76], except in the United States (US) and Sweden, where the peak already may have been reached [33]. Persons exposed to asbestos fibres in the past have a lifetime risk for developing MPM of 5-10% [77]. Nevertheless, given the latency period and the continued mining and use of chrysotile asbestos, it is expected MPM will remain a global health concern for future generations. Besides, there remains an environmental exposure to the fibres and recent documentation of a genetic familial predisposition due to BAP-1 mutations [46, 78].

Survival for MPM patients is very poor. Within one year after diagnosis, more than half of the patients has already died and the relative survival at five years after diagnosis is only 5% [14]. As shown in Figure 2 on page 10, Belgium has a standardized mortality for MPM and asbestosis of respectively 20 and 8 per million people per year in 2000-2004. Worldwide, the WHO

estimates 107,000 people die annually of asbestos-related diseases (lung cancer, mesothelioma and asbestosis) of whom 43,000 from MPM [79]. Next to this, 125 million people are still exposed to asbestos fibres at the workplace today and are potentially at risk for developing MPM.

3.2.2 Economic impact

Next to the direct substantial personal and health care burden for MPM, asbestos-related diseases will cost a large amount on extra compensation. It is estimated that the total economic burden of MPM will cost up to $200 billion for the US and $80 billion for Europe in the next 30 years related to compensation [15].

In Belgium, some patients with occupational asbestos-related diseases can obtain a compensation for their inability to work and the related medical costs. However, their condition has to be recognized by the Occupational Disease Fund (ODF), now fused to Fedris [80, 81]. For the other patients with mesothelioma or asbestosis who are not compensated by the ODF, the Asbestos Fund was founded in 2007 [82].

3.3 Diagnosing MPM

The onset of symptoms in MPM is often insidious and non-specific, shifting diagnosis to advanced stage disease in more elderly patients [33]. Symptoms like dyspnoea (due to pleural effusions) and chest pain (due to infiltration into the chest wall and its intercostal nerves) are the more prevalent in 88% of patients [20]. More suspicious symptoms like weight loss, fatigue, fever, thrombocytosis, hypo-albuminemia and anaemia do not appear at diagnosis, but tend to rise with advanced disease [15]. The diagnosis of MPM should be considered in any patient presenting with unilateral pleural effusion or thickening, especially if chest pain is reported [83]. For the diagnosis of MPM, several modalities can be explored.

3.3.1 Imaging techniques

The standard diagnostic work-up includes a chest X-ray to evaluate a pleural effusion or pleural thickening and a computed tomography (CT) scan of the chest and upper abdomen

[15, 33]. However, both should not be used alone for MPM diagnosis since the accuracy for MPM detection is hampering [14]. However, a rind-like tumour on CT along the pleural cavity together with diffuse or nodular pleural thickening are suggestive of the disease. Magnetic resonance imaging (MRI) is not yet relevant for the diagnosis of mesothelioma and is only recommended when tumour delineation is needed [84]. Positron emission tomography (PET) scanning is currently only useful for the staging of mesothelioma [33, 85, 86]. When radiological images are suggestive for MPM and a history of asbestos exposure is known, a cytological or histological analysis should be performed [87].

3.3.2 Cytology

Since more than 80% of MPM patients present with pleural effusions, cytological examination is often performed [88]. However, it is not recommended to establish a diagnosis of mesothelioma based on cytology alone because of its important false positive and negative rate [14, 89] and the fact that mesothelioma cells in the pleural fluid are always of the epithelioid type and do not show any typical aspects. Therefore, it is hard to make a differential diagnosis with benign, reactive mesothelial proliferations. Besides, cytology does not allow for evaluation of the invasiveness of the tumour. A histopathological examination of a pleural biopsy specimen obtained by thoracoscopy is required to establish a definitive diagnosis.

3.3.3 Histopathology

The gold standard to obtain a definite MPM diagnosis is the light microscopical evaluation of a histopathological tissue sample with additional use of a panel of immunohistochemical markers [33]. Thoracoscopy is preferred, allowing a diagnosis in more than 90% of cases, except in cases with pleural symphysis or contraindication to thoracoscopy, when either a surgical or transthoracic needle biopsy can be substituted, albeit with a lower sensitivity (±30%) [33].

In order to make a definite diagnosis, we first need to define the biopsy tissue as benign or malignant. Next, epithelioid mesothelioma needs to be separated from metastatic

adenocarcinoma. This can be done by using two markers with positive diagnostic value for mesothelioma (e.g. the nuclear markers anticalretinin and anti-Wilms tumour (WT) antigen-1 or the membrane markers anti-epithelial membrane antigen (EMA) for epithelioid mesothelioma, anti-cytokeratin(CK)5/6, antiD2-40 (podoplanin) or anti-mesothelin) and two markers with negative diagnostic value (e.g. a membrane marker like anti-Ber-EP4, a nuclear marker like antithyroid transcription factor-1, or monoclonal antigen markers like anti-carcinoembryonic antigen, anti-B72-3, anti-MOC-31, antioestrogen/progesterone) [89-91]. Among the various sources of antibodies, it is mandatory to use those presenting at a minimum of 60–70% sensitivity. It is not recommended to use anti-CK7/anti-CK20 to make the diagnosis of mesothelioma. Furthermore, to separate sarcomatoid mesothelioma from squamous and transitional cell carcinoma, it is recommended to use two broad-spectrum anti-cytokeratin antibodies and two markers with negative predictive value (such as anti-CD34 and anti-B-cell lymphoma 2 marker, anti-desmin, anti-S100) to confirm the diagnosis. Nevertheless, negative immunostaining with a single antibody does not exclude the diagnosis. With regard to atypical mesothelial hyperplasia (superficial mesothelial proliferations), there are currently no commercially available immunohistochemical markers that identify the benign or malignant nature of the cells observed. However, the discovery of inactivating mutations in the BAP1 gene has shown to be promising as marker to separate mesothelioma from reactive mesothelial proliferations [36, 92], although further confirmation is needed.

3.3.4 Staging

Staging is used to describe the anatomical extent of a tumour, correlates with prognosis and helps in selecting the optimal treatment strategy. The tumour stage is reported by using a TNM staging classification system. In this system, T (‘*Tumour’*) describes the extent of the primary tumour, N (*‘Nodes’*) evaluates the involvement of regional lymph nodes and M (*‘Metastasis’*) describes the presence or absence of distant metastasis.

For MPM, the recent 8th edition of the staging system developed by the International Association for the Study of Lung Cancer (IASLC), is recommended for use (Table 2) and is approved by the Union for International Cancer Control (UICC).

Accurate T staging is important in determining the resectability of the tumour. In patients with locally advanced tumours, imaging aims at distinguishing T3 from unresectable T4 disease. Contrast-enhanced CT is preferred for initial staging because of its easy accessibility and cost-effectiveness. Thoracic MRI is complementary to CT for identifying invasion of the chest wall, mediastinum, and diaphragm. However, PET with 2-deoxy-2-[fluorine-18]fluoro- D-glucose integrated with CT (^{18}F-FDG PET/CT) remains inadequate for accurately defining locoregional tumour extent [84, 93].

To assess the N status, ^{18}F-FDG PET/CT and thoracoscopy only showed moderate agreement for the presence of nodular lesions. Hence, CT is typically used to evaluate hilar and mediastinal nodal disease. However, the specificity of CT for detecting nodal disease is poor since occult metastases can be detected. Furthermore, ^{18}F-FDG PET is not suitable to assess nodal involvement because of the false-negative results in patients with microscopic disease as well as the false-positive results in patients with inflammatory or infectious conditions. These potential pitfalls can lead to misinterpretation and have implications for management [84, 93].

Distant metastases can be solitary or diffuse and may involve the brain, lung, bone, adrenal gland, peritoneum, abdominal nodes, and abdominal wall. Therefore, a whole-body ^{18}F-FDG PET/CT can be used to assess the M status [84, 93].

Table 2: 8th edition of the TNM classification for malignant pleural mesothelioma.

Descriptor/Stage		Extent of involvement
T	T1	Tumour involving the ipsilateral parietal or visceral pleura only
	T2	Tumour involving ipsilateral pleura (parietal or visceral) with invasion involving at least one of the following: • Diaphragmatic muscle • Pulmonary parenchyma
	T3[1]	Tumour involving ipsilateral pleura (parietal or visceral) with invasion involving at least one of the following: • Endothoracic fascia • Mediastinal fat • Chest wall, with or without associated rib destruction (solitary, resectable) • Pericardium (non-transmural invasion)
	T4[2]	Tumour involving ipsilateral pleura (parietal or visceral) with invasion involving at least one of the following: • Chest wall, with or without associated rib destruction (diffuse or multifocal, unresectable) • Peritoneum (via direct transdiaphragmatic extension) • Contralateral pleura • Mediastinal organs (oesophagus, trachea, heart, great vessels) • Vertebra, neuroforamen, spinal cord or brachial plexus • Pericardium (transmural invasion with or without a pericardial effusion)
N	NX	Regional lymph nodes cannot be assessed
	N0	No regional lymph node metastases
	N1	Metastases to ipsilateral intrathoracic lymph nodes (includes ipsilateral bronchopulmonary, hilar, subcarinal, paratracheal, aortopulmonary, paraoesophageal, peridiaphragmatic, pericardial, intercostal and internal mammary nodes)
	N2	Metastases to contralateral intrathoracic lymph nodes. Metastases to ipsilateral or contralateral supraclavicular lymph nodes.
M	M0	No distant metastases
	M1	Distant metastasis present
Stage IA	T1, N0, M0	
Stage IB	T2/T3, N0, M0	
Stage II	T1/T2, N1, M0	
Stage IIIA	T3, N1, M0	
Stage IIIB	T1/T2/T3, N2, M0 T4, N0/N1/N2, M0	
Stage IV	any T, any N, M1	

[1]T3 describes locally advanced, but potentially resectable tumour.
[2]T4 describes locally advanced, technically unresectable tumour.

3.4 Therapeutic strategies

MPM has a dismal prognosis with less than 5% five year survival [14]. When untreated, median survival of patients is 6-9 months. First-line treatment with standard of care chemotherapy provides a three month survival benefit [14] which even increases to 18 months after adding targeted therapy with bevacizumab, a monoclonal antibody against the VEGF receptor [94]. Nevertheless, MPM remains a fatal tumour despite the presence of several treatment options. Depending on the performance status of the patient, comorbidities, tumour stage and the patient's age, different treatment options can be chosen [95]. Around 40% of MPM patients respond to the combination of treatments [96-98]. For patients who are unresponsive to first-line treatment or became progressive after treatment, there is no standard second-line treatment. Furthermore, most of the patients present with unresectable disease, where palliative care to relieve the pain and dyspnoea is the only option. This includes thoracentesis, analgesia, pleurodesis, external beam radiation therapy and peritoneal-pleural or external shunting [47]. If the treatment can prolong survival and induce tumour response without significant toxicity, chemotherapy can also be used for palliative treatment. Next, we will discuss the possible treatment strategies for patients with MPM.

3.4.1 Radiation therapy

In the past, the instrument tracts following drainage or thoracoscopy were preventively irradiated as prophylactic radiation therapy to prevent the occurrence of metastasis in these tracts [99, 100]. However, its use is controversial since there is no evidence of reduced tumour seeding after this treatment [101, 102]. A recent randomized trial showed the lack of efficacy of prophylactic tract irradiation [103]. Nevertheless, in patients with painful infiltration or nodules, palliative radiation therapy can be used for pain relief [89]. Responses of more than 60% have been seen, although the duration of response is often disappointing (2-3 months). The presence of critical organs as the lungs, heart, liver spinal cord and oesophagus limit the use of radiation as radical treatment [104].

3.4.2 Chemotherapy

The current standard of care first line chemotherapy exists of the combination of an antifolate with platinum-derivates [33, 96, 105, 106]. The antifolate pemetrexed (Alimta™) and raltitrexed (Tomudex™) are multitargeted antifolate agents that inhibit multiple enzymes in the folate pathway and, hence, inhibit synthesis of purines and pyrimidines resulting in a reduced cell growth [107]. Pemetrexed induces haematological toxicity, which can be countered by adding folic acid and vitamin 12 supplements to the treatment [107]. As platinum derivates, cisplatin and its analogue carboplatin are the most commonly used where cisplatin has a higher toxicity profile [108, 109]. By using platinum, DNA strands are cross-linked and tumour cell death is induced by either apoptosis, necrosis or both [109]. A randomized phase III study of pemetrexed plus cisplatin versus cisplatin alone in chemo-naïve patients with MPM showed a response rate of 41.3% versus 16.7% and a median survival of 12.1 versus 9.3 months, in favour of the combination arm [96]. On the basis of the high response rate and the improvement in overall survival, pemetrexed in combination with cisplatin was approved by the United States Food and Drug Administration (FDA) in 2004 for the treatment of MPM patients who had unresectable disease or were unfavourable for curative surgery. Similar approvals were also granted by European regulatory agencies. Another study by van Meerbeeck *et al.* combined cisplatin with raltitrexed versus cisplatin and showed a response rate of 23.6% compared to 13.6% and a median overall survival of 11.4 months versus 8.8 months [110].

Unfortunately, 80% of patients have recurrent disease within the first 2 years after treatment. For second-line therapy, no general recommendations exist and patients are encouraged to be enrolled in clinical trials [33, 89]. Patients who had a partial or complete response with first-line chemotherapy may be treated again with the same regimen. If patients show progressive disease or experience severe toxicities, chemotherapy should be stopped. In stable or responding patients, up to six cycles can be given [33].

Several targeted therapies acting on several pathways in cancer have been investigated. The use of several small molecules inhibiting the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) like erlotinib and gefitinib or monoclonal antibodies against the EGFR receptor like cetuximab have not showed any response [105, 111]. Furthermore, imatinib and dasatinib, both inhibiting the tyrosine kinase activity from the platelet-derived growth factor

(PDGF) receptor, have not proven useful [112]. Another growth factor is vascular endothelial growth factor (VEGF) which plays a role in angiogenesis. VEGF Receptor inhibition by sorafenib, sunitinib, vatalanib, cediranib and thalidomide did not improve the overall survival for MPM patients [112]. However, as mentioned before, bevacizumab, an antibody binding VEGF, has been tested in a phase III trial in combination with cisplatin and pemetrexed. In patients who were able to receive bevacizumab next to standard of care, the overall survival was significantly longer (18.8 months) compared to standard of care cisplatin/pemetrexed combination (16.1 months) [94]. Another target is mesothelin, an antigen that is expressed on mesothelial cells and overexpressed in epithelioid mesothelioma (see Part I, Chapter 4). Amatuximab is a chimeric monoclonal antibody binding to mesothelin with great affinity. In a single-arm phase II study, cisplatin and pemetrexed were combined with amatuximab for six cycles, followed by amatuximab maintenance therapy in case of response or stable disease. Although the primary endpoint was not met, a partial response was seen in 39% of the patients and 51% of the patients had stable disease, underlining an activity of amatuximab in mesothelioma [113].

3.4.3 Surgery

Surgery can either be palliative or radical. Before performing any form of surgery, the patient's pulmonary and cardiac status needs to be properly evaluated [47].

Palliative surgery refers to debulking pleurectomy which consists of an incomplete macroscopic clearance of pleural tumour. The objective of this operation is to relieve the entrapped lung by removing the visceral tumour cortex. Removal of the cranial parietal tumour cortex may relieve a restrictive ventilatory deficit and reduce chest wall pain [33]. This operative procedure may be performed by either open thoracotomy, but the closed video assisted thoracoscopic surgery (VATS) is preferred. The associated morbidity of thoracotomy may diminish the benefits [114], however there is limited but emerging evidence that VATS can provide good symptom control and may have a beneficial effect on survival.

Radical surgery may be defined as an attempt to remove all macroscopic tumours from the hemithorax. These objectives are usually achieved by extrapleural pneumonectomy (EPP) with *en bloc* resection of visceral and parietal pleura, lung, pericardium and diaphragm and systematic nodal dissection followed by pericardial and diaphragmatic reconstruction with

prosthetic material [33]. Operative mortality has fallen to an acceptable level of 5% in experienced centres but morbidity remains high at 50% [115]. There is limited evidence for the efficacy of radical surgery for mesothelioma. However, EPP surgery alone for MPM is not curative since no free oncological resection margins can be obtained. The pleural lining, especially on the pericardium and mediastinum cannot be resected with a 1–2 cm margin [116]. Extended pleurectomy/decortication (e-P/D) is another surgical procedure, which aims at maximal debulking with resection and reconstruction of the diaphragm and/or pericardium if necessary [117]. An e-P/D can involve resection of a lobe, multiple wedges, or even a segment as long as it is lung sparing. There is less mortality and morbidity in comparison with EPP, but the procedure currently suffers from a lack of standardisation and uniformity. Consequently, there is a high risk for recurrence and this observation is therefore the rationale for combined therapy.

3.4.4 Multimodality approach

Since several studies have shown that surgical treatment alone offers dismal prognosis only [118, 119], multimodality approaches including chemo- and/or radiotherapy have been suggested in order to improve survival. The best long-term results are achieved by multidisciplinary strategies that include EPP together with either neo- or adjuvant chemotherapy [115, 116]. The particular advantage of chemotherapy before surgery (neoadjuvant) is a possible reduction of tumour load and downstaging, which may lead to a better understanding of the individual tumour biology and a more optimal patient selection. A median survival time of about 2 years has been reported by several studies, including some long-term survivors [115, 120]. Nevertheless, these trials included highly selected patients with the best prognosis, younger age, and most often only the epithelioid subtype. The Mesothelioma and Radical Surgery (MARS) trial demonstrated the feasibility of randomizing patients for chemotherapy alone or followed by EPP [121, 122]. The trial found no evidence that patients would benefit from EPP. Importantly, it must be stated that the study was underpowered to prove the effectiveness of EPP. The bulk of data supporting EPP are derived from a series of uncontrolled studies on subsets of highly selected patients. The lack of encouraging survival with EPP is reflected by studies on patterns of recurrence, which are mostly in the ipsilateral hemithorax. Furthermore, the choice between EPP and e-P/D is to be

elucidated. A study that compared EPP versus e-P/D demonstrated better survival associated with e-P/D than with EPP after controlling for histologic type, stage, multimodality treatment, and sex [123]. Despite potential bias due to the retrospective nature of this study, surgeon selection, and variations in adjuvant treatment, the surgical numbers are large enough to allow comparison. If EPP is to be preferred despite its higher operative risk, then huge differences in survival are to be expected. However, such differences were not observed. In practice, e-P/D preserves more lung tissue and demonstrates similar or better overall survival and decreased postoperative morbidity and mortality when compared with EPP, while patterns of recurrence remain local. E-P/D appears to provide the most benefit and the least harm to the patient with mesothelioma.

Since e-P/D has been associated with similar to better outcome than EPP, and EPP accompanied by perioperative chemotherapy and radiotherapy was better than EPP alone, the question arises whether e-P/D as part of a multimodality treatment further improves outcome. Therefore, the role of e-P/D will be investigated in a "MARS-2" trial. In this trial, e-P/D will be added to standard induction chemotherapy and randomly compared with chemotherapy only [119]. The European Organisation for Research and Treatment of Cancer (EORTC) is presently conducting a randomised controlled trial whereby patients are allocated to e-P/D preceded or followed by chemotherapy (www.clinicaltrials.gov, identifier NCT02436733).

Because of its negative effects, postoperative radiotherapy (PORT) is not recommended in cases where the lung remains *in situ* after decortication. The introduction of intensity-modulated radiotherapy (IMRT) seems to overcome most of these issues and allows the remaining tissue to be properly irradiated or use it as neoadjuvant therapy.

3.4.5 Immunotherapy

Based on preclinical studies and clinical trials, the role of immunotherapy in cancer treatment has rapidly increased. MPM has a high infiltration of lymphocytes (TILs) and macrophages and a significant T-cell inflammatory expression pattern [124]. The tumour escape mechanisms have different ways to elude immune reaction, including the expression of T cell inhibitory ligands, such as cytotoxic T-lymphocyte antigen 4 (CTLA4), programmed death 1 ligand (PD-

L1), and programmed death 1 receptor (PD-1), making these interesting as therapeutic targets for immunotherapy.

PD-L1 expression in MPM was correlated with disease extension at presentation and with sarcomatoid subtypes. Immune checkpoint inhibitors resume T cell cytotoxic activity on cancer cells by blocking T cell inhibitory mechanisms. Monoclonal antibodies like ipilumumab and tremelimumab bind the CTLA4 receptor, whereas pembrolizumab and nivolumab bind PD-L1 and PD-1 receptor, respectively. Tremelimumab is a fully human IgG2 anti-CTLA-4 antibody and was tested in a phase II study in MPM patients that progressed after first-line platinum-based therapy [125]. The most common side effects with tremelimumab were gastrointestinal toxicity (colitis and diarrhoea) and dermatological toxicity (rash and pruritus). The trial didn't reach the primary end point but the disease was controlled in 31% of patients, with 6.2 months of progression free survival (PFS) and an overall survival (OS) of 10.2 months. These data suggested promising activity in MPM. Considering anti-PD-1 and anti-PDL1 antibodies, pembrolizumab demonstrated to be safe and tolerable for patients with MPM in a phase Ib trial (KEYNOTE-028) that included several types of solid tumours. Preliminary analysis showed a 28% of patients had a partial response where 48% of patients remained stable. The overall disease control rate was 76%. These new developments in immunotherapy will open new perspectives for treatment.

A relatively new field of therapy are tumour vaccines [105]. Vaccines against mesothelioma cells may increase immune responses against the tumour in which patients receive mature dendritic cells (DCs), pulsed with the patient's own tumour lysate, after chemotherapy. In pilot studies, treatment was feasible and safe and in some patients anti-tumour immune responses were detected [126]. A study showed that the WT1 peptide vaccine gave minimal toxicity and induced immune responses against WT1 in a high proportion of patients (78%) [127]. Therefore, a study loaded DCs with mRNA encoding the WT1 protein and injected these intradermally in 10 patients with unresectable, epithelial MPM and non-progressive disease after standard of care chemotherapy [128]. Seven patients showed stable disease and 3 had progressive disease. Median overall survival (OS) from start of chemotherapy was 32 months; this compares with an OS of 22 months reported in the literature for a similar subgroup of patients treated with chemotherapy only. However, this should be further tested in a larger number of patients and a clinical phase I/II trial is currently recruiting patients to test dendritic

cells (DCs) loaded with the mesothelioma-associated tumour antigen WT1 in conjunction with conventional chemotherapy for the frontline treatment of resectable MPM (clinicaltrials.gov identifier NCT02649829).

3.5 Screening for MPM

As mentioned, the WHO estimates that annually 125 million individuals are still exposed to asbestos fibres at the workplace and are potentially at risk for mesothelioma [79]. Therefore, screening these asymptomatic persons at risk would be beneficial if the detection of the disease is possible at an earlier stage and, hence, improves the prognosis by more effective treatment options, assuming that therapy is more effective if given at earlier stage [33, 129]. By doing so, persons with a positive test could be subjected to a further more intensive surveillance with imaging techniques or undergo a thoracoscopy. In that way, not every individual at risk will be subjected to these procedures, limitating the cost and associated screening radiation if screening was proposed to asbestos-exposed subjects. For MPM, a biomarker with a positive predictive value (PPV) of 10% is considered acceptable for screening [129]. However, given the rather low prevalence of MPM, specificity then needs to be very high (>95%) since otherwise the number of false-positive subjects would be several times higher than true-positive subjects which would then all undergo unnecessary diagnostic procedures [130].

As will be discussed in chapter 4, the available data for MPM about the performance of potential screening biomarkers hampers a large-scale screening program up to today. There are no studies that recommend the screening of persons at risk with an (occupational) history of asbestos exposure [89]. Considering imaging techniques, low-dose computed tomography (LDCT) scans have not proven to be effective for the early detection of MPM [131]. Furthermore, a PET scan and MRI are useful for MPM management and in the differentiation of malignant from benign pleural disease, but are not applicable for screening purposes. Next to the high cost, the associated radiation exposure also limits its further use [77, 129, 131]. Several blood biomarkers will be discussed in the next chapter but a recent meta-analysis assessed the value of serum mesothelin-related protein (SMRP) as a screening tool (chapter 4, paragraph 4.2). Including 4,491 individuals of which 1,026 were MPM patients, the sensitivities and specificities of SMRP ranged from 19%-68% and 88%-100%, respectively

[132]. The poor sensitivity and PPV (14%-46%) of mesothelin clearly limits its value as early marker for MPM diagnosis and stresses the need for further biomarker research.
Lastly, there is no proof that early discovery of MPM would cure the patient or even improve their survival. Therefore, prospective studies should investigate the effect of earlier treatment on survival.

CHAPTER 4
BIOMARKERS

4.1 Introduction

In 1998, the National Institutes of Health (NIH) Biomarkers Definitions Working Group defined a biomarker as *"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"* [133, 134]. There are different types of biomarkers depending on their clinical use [135]: those that assist in the care and follow-up of asymptomatic patients (screening biomarkers), biomarkers to identify patients in subjects suspected to have the disease (diagnostic biomarkers) and those that can predict the prognosis in treatment-naïve patients with overt disease (prognostic biomarkers). Biomarkers can also be used for predicting the treatment response (predictive biomarkers) or for surveillance after therapy (monitoring biomarkers). Fundamental for the use of biomarkers in all situations is the biomarker's accuracy or the ability to correctly classify one condition and/or outcome from another (for instance healthy versus diseased).

Although different kinds of biomarkers exist, blood proteins are being studied the most because these manage and regulate many catalytic processes and structural and signalling functions in living organisms [136, 137]. Furthermore, blood is considered to be the most comprehensive and represents all body tissues. Also, blood sampling is less invasive for the patient with the medical infrastructure immediately available at the hospital site [137, 138]. By measuring their responses, changes and malfunctions in the metabolism and disease progression can be explored.

4.2 Blood biomarkers

Blood exists out of 55% plasma while the remaining 45% consists of blood cells (platelets, white blood cells and erythrocytes) [139]. After plasma clots, serum remains, which is similar to plasma but has lost fibrinogen and has prothrombin cleaved to thrombin [136]. Several biomarkers have been studied in blood. One of the most extensively evaluated serum

biomarkers is soluble mesothelin-related protein (SMRP). Several studies have shown SMRP levels to be significantly increased in MPM patients, making this biomarker interesting to investigate as a screening tool. *Ferro et al.* studied SMRP levels in both serum (S-SMRP) and pleural effusions (PE-SMRP) [140]. They found that PE-SMRP had a better diagnostic performance in differentiating MPM from other malignancies and asbestos-related benign diseases. Despite of the high specificity, S-SMRP shows a lack of sensitivity. Therefore, further research has focussed on the combination of serum mesothelin with several other biomarkers in order to improve the diagnostic accuracy. *Creaney et al.* combined SMRP with cancer antigen 125 (CA125) but this did not improve sensitivity for detecting MPM over SMRP alone [141].

Osteopontin (OPN) and megakaryocyte potentiating factor (MPF) are also biomarkers that show increased levels in MPM patients. The diagnostic performance of these markers was assessed in multiple studies but both glycoproteins lack sensitivity to be used as stand-alone diagnostic biomarkers [142-147]. However, combining SMRP and OPN improved the diagnostic accuracy [145] opposed to a previous study where the combination of SMRP with OPN did not improve the diagnostic performance compared to SMRP alone [142]. The same has been observed for the combination of SMRP and MPF [142].

It has been shown that SMRP levels correlate with the histological subtype of the tumour since it is only expressed in epithelial mesothelioma. The same correlation has been observed for MPF [148]. This is due to the fact that SMRP and MPF both originate from the same mesothelin gene [91]. Mesothelin encodes a 69 kilodalton (kDa) precursor protein which is bound to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor [149]. After glycosylation, this precursor is cleaved into the 40 kDa membrane-bound mesothelin and the 31 kDa glycoprotein MPF. When the GPI anchor is cleaved, mesothelin enters the bloodstream as ‘soluble mesothelin-related protein’ (SMRP). Membrane-bound mesothelin is normally expressed on normal mesothelial cells, and highly expressed in cancers like pancreatic cancer, ovarian cancer and epithelioid mesothelioma. Asbestos-exposed individuals seem to have higher SMRP concentrations than normal control individuals, regardless of the presence of pleural disease. Therefore, serum SMRP levels also can be a marker of asbestos exposure [150].

Other interesting biomarkers are C-C motif chemokine ligand 2 (CCL2) and galectin-3, both measured in pleural effusions [151]. CCL2 levels were found increased in MPM patients, where

galectin-3 concentrations were found to be decreased in case of MPM [151]. Furthermore, the combination of SMRP, CCL2 and galectin-3 had better diagnostic accuracy for MPM compared to SMRP alone [152].

Another potential diagnostic marker is thioredoxin-1 (TRX), which was found elevated in MPM patients in comparison with asymptomatic asbestos-exposed individuals [153]. Research also focussed on fibulin-3 as a potential diagnostic biomarker. Fibulin-3 levels in plasma and pleural effusions were able to distinguish MPM patients from controls [154]. Although this was on retrospective data and the marker had a similar diagnostic performance as SMRP [155, 156], SMRP was later shown to outperform fibulin-3 [157].

Very recently, it was shown that the levels of total HMGB1 in serum discriminated asymptomatic asbestos-exposed individuals from non-asbestos-exposed healthy subjects [158]. A specific HMGB1 isoform (hyper-acetylated HMGB1) even outperforms previously described biomarkers and discriminated between MPM from asbestos-exposed or non-exposed individuals with 100% sensitivity and specificity [158]. Combining fibulin-3 with either total or hyper-acetylated HMGB-1 improved both sensitivity and specificity for differentiating MPM patients from individuals with non-MPM pleural effusions [158]. Although some of the abovementioned biomarkers show diagnostic potential, none of these are validated and therefore cannot be used in clinical practice to screen potential individuals at risk for MPM. This stresses an urgent need to continue the search for an accurate diagnostic biomarker to enable early stage diagnosis of MPM. Therefore, the field of breath analysis can be explored.

CHAPTER 5
BREATHOMICS FOR ASBESTOS-RELATED DISEASES

Breath contains volatile organic compounds (VOCs) which arise from the (patho)physiological processes of the body [159, 160]. The generation of VOCs is linked to oxidative stress and increased metabolism which releases VOCs from the tissues into the blood stream and which are transported through the circulation to the lungs [161-164]. Volatile compounds will undergo the gas-exchange mechanisms in the alveolar region of the lungs and, hence, enter the breath. In that way, breath can be a non-invasive source of biomarkers for disease and is a less complex mixture than blood. Chapter 5 focusses on the different aspects of human breath, the different techniques used for breath analysis and current state-of-the-art discussing breath analysis for the spectrum of asbestos-related diseases.

Strengths, weaknesses, and opportunities of diagnostic breathomics in pleural mesothelioma - a hypothesis.

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ABSTRACT

Past and present asbestos use will reflect in increasing numbers of mesothelioma cases in the next decades, diagnosed at a late stage and with a dismal prognosis. This stresses the need for early detection tools which could improve patient's survival. Recently, breath analysis as a non-invasive and fast diagnostic tool has found its way into biomedical research. High-throughput *breathomics* uses spectrometric, chromatographic and sensor techniques to diagnose asbestos-related pulmonary diseases based upon volatile organic compounds (VOCs) in breath. This article reviews the state-of-the-art available breath analysing techniques and provides the insight in the current use of VOCs as early diagnostic or prognostic biomarkers of mesothelioma in order to stimulate further research in this field.

Introduction

Asbestos fibres cause malignant pleural mesothelioma (MPM), an aggressive tumour from the serosal surface lining the pleural cavity [14]. Because of the large asbestos consumption in the past, MPM incidence rates will further increase the next decade [2, 13], although with large intercountry differences [15, 132], and peak between 2015-2020 [14, 33]. This MPM epidemic will cost the United States and the European Union $200 billion and $80 billion respectively in compensation, urging the need for an early detection [77].

The 'Holy Grail' in MPM diagnosis would be a non-invasive, accurate test in asbestos-exposed persons at risk for developing MPM which makes early stage detection possible, reduces the economic burden of wild screening and improves MPM management. Present research efforts have however, not yet revealed a validated diagnostic blood biomarker [132, 154].

Breathomics is an innovative, non-invasive tool that uses high-throughput technologies to identify organic molecules as biomarkers that reflect the patient's metabolic status. The aim of this manuscript is to critically review the current state of the art concerning different techniques used for gaseous breath analysis, focused on their diagnostic yield in various asbestos-related diseases and MPM specifically in order to stimulate future MPM biomarker research using breathomics and improve MPM management.

Search strategy and literature selection

MEDLINE (PubMed) and Web of Science were searched for studies concerning exhaled breath research in asbestos-related pulmonary diseases until December 2013. Following keywords were selected as MeSH terms and used as search terms in PubMed and Web of Science: "asbestos", "asbestosis", "mesothelioma", "lung neoplasms" and "breath tests". Other additional search terms included "breath analysis", "pleural plaques", "pleural thickening", "pleural effusion", "volatile organic compounds" and "lung cancer". After a first inspection of the literature "electronic nose", "ion mobility spectrometry", "GC-MS", "exhaled breath condensate" and "dog" were added as additional terms for specific search combinations. Only literature published in English on human material was considered. References of the 131 selected publications were searched manually for additional relevant literature concerning breath analysing techniques in asbestos-related diseases. This delivered another 21 publications.

The spectrum of asbestos-related diseases

Asbestos fibres are hydrated silicate minerals containing a high tensile strength and resistance to chemical and thermal degradation, making these 'magic minerals' interesting for insulation in (ship) building [165]. Hence, many people were professionally exposed to the fibres. Inhaling asbestos fibres damages the lung parenchyma and the pleura, leading to benign conditions like pleural effusions, pleural plaques, diffuse pleural thickening (DPT) and asbestosis but also to malignant diseases like lung cancer (although in association with tobacco) and MPM (Figure 7). The World Health Organization estimates that annually 125,000 new persons are confronted with an occupational asbestos exposure [79] and worldwide, there are approximately 25,000 patients diagnosed with mesothelioma per year [166]. The lifetime MPM risk after occupational asbestos exposure is 10% [77] with a mean latency period between exposure and diagnosis of 40 years (range 15-67 years) [33]. A non-specific symptomatology and a lack of imaging accuracy delay its diagnosis so that patients present in an advanced stage which jeopardizes potential curative management [14]. Despite that current guidelines dissuade screening for MPM [77], asbestos-exposed individuals are wildly and widely subjected to non-specific investigations (lung function testing, chest X-rays and computed tomography scans) at an unknown cost [33].

Asbestos exposure also increases the likelihood for lung cancer development through a synergistic effect with smoking and via the mechanism of fibrosis [167, 168]. Any biomarker of asbestos-induced lung cancer is hence likely to be confounded by smoking. Happily, a low-dose spiral CT scan has recently been advocated an effective technique for the early diagnosis of lung cancer [169, 170] but not of mesothelioma [171, 172], urging the need to search for other early detection tools for the latter disease.

Breath analysis: techniques and applications

Exhaled breath is easily and non-invasively retrievable, resembles the arterial concentrations of biological substances and can be monitored in real-time. It consists of a liquid phase containing water and proteins and a gaseous phase containing oxygen, nitrogen, carbon dioxide, water, inert gases, volatile organic compounds (VOCs) and non-VOCs [173-175]. Since their first discovery, more than 3000 VOCs have been detected in breath [176], arising from exogenous sources (via inhalation or skin adsorption) or from endogenous biochemical processes (via oxidative stress, fat metabolism) and are catabolized through cytochrome P450

enzymes. Independent of their origin, VOCs are transported through the blood to the lungs where they enter the breath by the alveolar gas exchange mechanisms. Hence, changes in the VOC metabolism result in different VOC profiles in breath [177].

Oxidative stress plays a key role in endogenous VOC production: acetone is linked to lipolysis via decarboxylation of excess acetyl-CoA after oxidation of fatty acids, isoprene is liberated during the mevalonic pathway of cholesterol synthesis and hydrocarbons are formed through lipid peroxidation of polyunsaturated fatty acids in the cellular membranes [178]. Since asbestos-related diseases are driven by oxidative stress and inflammation, markers of oxidative damage, such as VOCs, are hence likely to be altered. Asbestos fibres have indeed a high iron content which directly induces oxidative stress generating reactive oxygen species (ROS) and nitrogen species (RNS) [51, 179]. Because of the asbestos fibre's high length/width ratio, alveolar macrophages fail to engulf them, leading to a 'frustrated phagocytosis' [180] (Figure 7). In this way, asbestos indirectly induces the release of oxidants, cytokines and growth factors by the macrophages (and to a lesser extent by the mesothelial cells). This sustains the relentless generation of ROS and RNS which leads to (i) lipid peroxidation generating saturated hydrocarbons and aldehydes, (ii) protein oxidation and (iii) mutagenic DNA lesions through the generation of 8-oxo-7,8-dihydro-2'-deoxyguanosine and 8-nitroguanine [181, 182]. In addition, asbestos-induced mesothelial cell death is also linked to the inflammatory reaction associated with asbestos carcinogenesis [55] and hence could play a role in VOC production. When asbestos damages the mesothelial cells, High Mobility Group Box-1 (HMGB1) is released, inducing the accumulation of macrophages, inflammation and the release of tumour necrosis factor-α (TNF-α) [183, 184], which binds its induced receptor on the mesothelial cells. This activates the NF-κB pathway, increasing the survival of damaged mesothelial cells and aiding MPM pathogenesis [55].

Several very sensitive breath analysing techniques are now available for the liquid or gaseous phase of the breath and measure one of the different (non-)volatile breath compounds or recognize VOC patterns.

A) Analysis of the liquid phase

Exhaled breath forms a condensate with water representing over 99% of the exhaled breath condensate (EBC) sample and contains different molecules ranging from simple ions to DNA [185, 186]. Although not the scope of this review, diagnostic markers of asbestosis, pleural

plaques and diffuse pleural thickening in EBC have been investigated (Table 3). The non-volatile oxidative markers H_2O_2, 8-isoprostane, 4-hydroxy-*trans*-2-nonenal, 8-OHdG and the leukotrienes (LT) B4, D4 and E4 were found increased in the breath of asbestos-exposed individuals or patients with asbestos-related diseases compared to healthy controls [187-194]. However, no EBC measurements have been performed for MPM specifically. LTB4 is a potent chemotactic factor for neutrophils and asbestos exposure has been shown to provoke LTB4 secretion in alveolar macrophages *in vivo* [195, 196]. These findings suggest that the LTB4 level in EBC reflects the inflammatory response to asbestos and is attractive for further research in MPM. The EBC-level of 8-isoprostane is related to the degree of oxidative stress, tissue damage and fibrosis and hence, could also be used as a marker for lung cancer or could be confounded by smoking.

B) Analysis of the gaseous phase

Endogenous volatile metabolic products derived from the tumour or its environment and present in the gaseous phase of breath are of interest because they reflect tumour-specific biomarkers that are linked to their pathogenesis.

Nitric oxide (NO) serves as a signalling molecule maintaining physiological control of airway function [197]. Asbestos induces chronic lung inflammation in which an inducible form of NO-synthase (iNOS) is upregulated and the pulmonary gas diffusion capacity is decreased. Hence, the fractional exhaled NO (FeNO) in the gaseous phase of the breath of asbestos-exposed individuals increases. This was seen in asbestosis patients compared to those with pleural plaques, DPT and healthy controls [198] (Table 3). When flow rates over 250 ml/min are achieved, alveolar NO tends to be higher in asbestosis patients [187, 189] and in asbestos-exposed persons with borderline high-resolution CT (HRCT) parenchymal changes [194]. This reflects the ongoing lower respiratory tract inflammation and it seems that alveolar NO is related to the degree of pulmonary fibrosis rather than to the asbestos exposure per se. Hence, the increased production of NO is not tumour-specific, is attributed to tumour-associated non-specific immunologic and (asbestos-related) inflammatory mechanisms and correlates with the intensity of NO-Synthase 2 expression in alveolar macrophages [199].

Although NO levels are dependent of age, sex and lung function, FeNO determination in early MPM diagnosis can have a role which has not been investigated until today. The most popular

technique in determining FeNO is chemiluminescence, although handheld devices with electrochemical sensors are available, such as NIOX MINO® (AeroCrine AB, Solna, Sweden). Advances in detection systems resulted in (i) combinations of mass spectrometers and fast flow tubes such as proton-transfer-reaction mass spectrometry and selected ion flow tube MS, (ii) spectroscopic methods for identification and quantification of small molecules such as ion mobility spectrometry (IMS) and (iii) non-specific electronic noses (eNoses) that 'smell' gas combinations. All these methods provide the capability of easily gathering repeated samples and obtaining results immediately in a relatively short period of time.

Gas Chromatography (GC)-Mass Spectrometry (MS)

GC-MS detects and quantifies gases from 100 parts per million to 1 part per billion by separating compounds based upon their elution times from the GC-column and characteristic fragmentation pattern obtained by MS (Figure 8A). Today, only one reported GC-MS analysis of the breath of respectively 13 MPM patients, 13 healthy asbestos-exposed individuals and 13 healthy controls [200]. Table 4 summarizes the 18 VOCs identified as being able to discriminate MPM breath from controls with most of them being alkanes. The authors found higher cyclohexane concentrations in the breath of MPM patients compared to the other control groups in which a model they have built distinguished these groups with 97% accuracy. However, cyclohexane and several other VOCs were also found in the breath or urine of lung cancer patients and in *in vitro* studies with sensitivities and specificities ranging respectively from 71-86% and 66-100% [201-211] (Table 4). This indicates that these VOCs originate from oxidative stress in the inflamed stroma and hence, lack specificity for MPM since they are also seen in other cancers like lung cancer. Since asbestos can also induce lung cancer [29], non-specific inflammation-related VOCs in lung cancer patients can be of great interest to investigate in MPM and vice-versa. Two reports identified 2-methylpentane as a potential marker for lung cancer [210, 211] which was also found in *in vitro* tests [212]. Increased concentrations of C_4-C_{24} hydrocarbons [213, 214] and C_3-C_9 aldehydes [215] reflecting oxidative stress and lipid peroxidation, were also found in lung cancer patients and represent also promising markers as inflammation-related VOCs in MPM.

Ion Mobility Spectrometry (IMS)

IMS allows separation of VOCs in the breath by their ion mobilities (size, mass, shape, charge) [216, 217]. Gaseous metabolites are firstly ionized in an ionization chamber by a low energetic ^{63}Ni β-radioactive source, although other sources exist such as UV-light, lasers, electrical discharges or electrospray, followed by a separation in a drift tube under influence of a counter gas (Figure 8B). Conventional IMS is based on absolute ion mobilities measured at low electrical fields while other IMS-based methods, as differential IMS or field asymmetric waveform IMS, use the mobility difference at high and low electrical field, eliciting a periodic asymmetric waveform [218]. Combining IMS with multicapillary GC-columns (MCC) permits an effective preseparation of VOCs based upon their chemical characteristics before entering the ionization chamber and drift tube (Figure 8B). A direct online measurement using 10 mL of human breath and a total analysis time of 10-15 min makes MCC/IMS utterly suitable for clinical applications.

Data from MCC/IMS in patients with MPM or asbestos-related disease have presently not been reported, except one where 25 male patients with asbestosis or diffuse pleural thickening were discriminated from healthy controls with 96% sensitivity, 50% specificity and a respective positive (PPV) and negative (NPV) predictive value of 80% and 86% based upon alpha-pinene and 4-ethyltoluol [219]. Furthermore, its potential is demonstrated in 3 studies reporting breath analysis in lung cancer patients. Westhoff *et al.* identified 23 peak regions discriminating lung cancer patients from healthy controls with zero percent error rate and PPV and NPV of 100%, independently of smoking status, TNM-stage and histology [217]. However, their lung cancer patients were older and at an advanced stage and the healthy controls were not age-matched. Another study compared bronchoscopically obtained tumour-side air with non-tumour side air wherein n-dodecane discriminated lung adenocarcinoma patients from controls with 100% sensitivity, 75% specificity, 80% PPV and 100% NPV [220]. 2-butanol or 2-methylfuran and nonanal discriminated squamous cell carcinoma from healthy controls with sensitivities of 78-79%, specificities of 67-78%, PPV's of 70-80% and NPV's of 75-88%, which was confirmed more recently [221]. Although to minimize confounding, each patient with a tumour was its own control and healthy subjects were not included, 2-butanol and nonanal had a higher value in the tumour-bearing lung but no correlation of the VOCs with the location or the stage of the tumour was found.

Electronic noses (eNoses)

Electronic noses are based upon the change in surface conductivity of non-selective sensors when exposed to a bulk of different volatile breath compounds. These detectors recognize breath patterns without identifying and quantifying these compounds. VOCs induce a physical or chemical sensor change that sends a signal to a computer which then makes a classification. By analysing the breath of several patient groups and building a model based upon several data mining techniques, eNoses can be trained to identify a mixture of VOCs as a 'smellprint' (Figure 8C). By generating a database of breath signatures, identification of subjects with similar breath chemical characteristics becomes possible. Different sensors can be used as eNose sensors [222]. The most frequently used eNose, the Cyranose 320 (Intelligent Optical Systems, Inc., CA, USA), is a commercial and portable system whose detection capacity consists of an array of 32 individual polymer sensors blended with carbon black composite.

Dragonieri *et al.* [223] were the first to describe the use of an eNose in diagnosing MPM. They distinguished 13 MPM patients from 13 asbestos-exposed individuals and 13 healthy controls with respectively 92% sensitivity and 86% and 69% specificity. This study was repeated, now distinguishing 20 MPM patients from 18 asbestos-exposed and 42 healthy individuals with 90% sensitivity and 88% specificity [224].

An *in vitro* study used an eNose to compare the headspace air between cell lines for mesothelioma, non-small cell lung cancer, metastatic squamous cell carcinoma and healthy controls [225]. eNoses separated the different malignant cell lines and discriminated malignant from non-malignant cell lines. These two studies underline the potential of eNose analysis, next to GC-MS analysis, for detecting and diagnosing MPM.

Canines

Since eNose pattern recognition is based upon natural olfactory perception, dogs have been used to discriminate different breath patterns. Due to their sensitive smelling ability and learning capacity, several dog stocks were trained to recognize and discriminate different breath samples. Although no studies using canine scent for asbestosis or mesothelioma are available, lung cancer patients have been distinguished from controls with a 71-83% sensitivity and 82-93% specificity [226, 227]. However, bias in these studies result from confounding smoking, drugs or the use of different dog stocks.

Properties of breath analysing techniques

An ideal breath analysing method should be cheap, user-friendly, non-invasive and easy accessible without patient discomfort or traveling. The above described techniques developed for gaseous breath analysis, all have their specific advantages and disadvantages as summarized in Table 5.

Dogs, eNoses and IMS have a high sensitivity and are transportable but do not identify the individual compounds. Offline sampling methods (like GC-MS or eNoses) use different bags and tubing material for breath sampling, are not in real-time and induce a risk of sample contamination which leads to potential biases for data comparison between research groups. The water content of breath samples is also pernicious for sensor analysis, as it causes sensor drift and leads to possible loss of information by dilution. The sampling technique must handle this by removing the water vapour before the analysis by preconcentration, increasing the risk of contamination and losing some of the VOCs. When offline methods are used, mixed breath is stored in bags or tubes which can furthermore induce errors.

GC-MS analysis remains the gold standard, as it is very sensitive and allows VOC identification and quantification, albeit very expensive, time-consuming and laborious and it requires a large vacuum-containing set-up and qualified technicians. Furthermore, in conventional mass spectrometers, the impact of electrons on polyatomic molecules and complex gas mixtures as breath induces large fragmentation of the molecules. This complicates the mass spectra interpretation and makes it practically impossible to carry out accurate quantitative analyses. A more desirable technique is one in which the patient breathes directly (online) into the analytical equipment without any preconcentration steps and achieves the ionization of these gases with minimal fragmentation of the molecules which simplifies the outcome. This gives fast, real-time information about the breath composition, allows for instantaneous feedback and decreases the chance of contamination. IMS coupled to an MCC has these features (Table 3) and subjects breathe directly into a CO_2-controlled end-tidal breath collecting device collecting alveolar air in a sample loop followed by a fast analysis. A VOC database comprising correlated GC-MS with IMS spectra, allows immediate VOC identification and the effective separation of humidity is an advantage.

Conclusion and future directions

Past and current asbestos consumption will lead to more cases of mesothelioma in the coming decade. Breath analysis as a non-invasive diagnostic tool provides a fast, user-friendly and cost-effective system which is not subjected to laboratory conditions. Hence, breathomics can become the missing link to screen for early cases in professional or environmental asbestos-exposed persons i) to rule out diagnosis, requiring a high NPV, ii) to rule in diagnosis, necessitating a high PPV and iii) as a step-up diagnostic tool in order to enrich the fraction of screenees for further testing, requiring a low false negative rate.

Although they are not tumour-specific, one of the most abundant detected molecular groups in the breath of asbestos-exposed individuals are alkanes representing the oxidative stress level (Table 4). Even though published studies were able to discriminate asbestos-related diseases from healthy controls, there is a lot of variation in sample size, study design, control groups, techniques, sampling procedures, the kind of sampled breath (mixed/alveolar), preconcentration techniques and data-mining techniques. This makes it hard to compare the studies or draw clinically relevant conclusions at the present time.

Nevertheless, breath analysis will eventually become a useful tool for the diagnosis of asbestos-related diseases but the way to a validated and clinical implemented model is still harsh and cumbersome. A large, prospective, case-control study is to be performed using control groups with comparable characteristics that cover the possible confounding factors for asbestos-related diseases in order to strengthen the diagnostic tool's discriminative power. Therefore, healthy, non-smoking and smoking controls should be included as well as healthy asymptomatic asbestos-exposed individuals and participants with benign asbestos-related diseases. Lung cancer and mesothelioma patients should also be included, preferable treatment-naive and their VOCs should be analysed by disease stage. Standardization of the sampling techniques is necessary to allow comparison with results obtained in the field. Models should be built based upon a training set and internally validated in a separate validation set accounting for ambient air, gender, smoking behaviour and age. External validation should be performed in an independent test set estimating the general error. Different data mining techniques (classification trees, discriminant analysis, support vector machines, random forests, principle component analysis) should be investigated in cooperation with (bio-)statisticians [228]. In order to select appropriate VOCs representing MPM, comparisons with cell lines or xenografts and pre- and postoperative VOC analysis

should be performed. However, the importance of identifying different compounds is debatable: from pathophysiological view, VOCs should be identified with MCC/IMS or GC-MS to link MPM-related VOCs to their specific pathogenesis and hence allow to investigate therapeutic targets. On the other hand, recognizing breath patterns with dogs or eNoses allows for a clinical diagnostic assessment and monitoring.

Finally, an international consortium, discussing and optimizing each technique in large-scaled validation studies is needed and therefore an International Association for Breath Research (IABR) Task Force for breath sampling was recently founded. This could increase the speed of developing good diagnostic methods in asbestos-related diseases in a cost-effective way.

Table 3: Overview of FeNO and of breath biomarkers measured in EBC in asbestos-related diseases.

Marker	Disease (n)	Control subjects (n)	Method	Result	Ref.
Oxidative stress					
Hydrogen peroxide (H_2O_2)	Asbestosis (18) Pleural plaques (26) DPT (16)	Healthy (26)	EcoScreen + EIA	Asbestosis > HC	[187]
8-isoprostane	Asbestos-exposed (92)	Healthy (46)	SPE + LC-ESI-MS/MS	Asbestos-exposed > HC	[191]
	Asbestos-exposed (44)	NA	SPE + LC-ESI-MS/MS	Asbestos-exposed > HC	[193]
	Asbestos-exposed (45)	Healthy (29)	SPE + LC-ESI-MS/MS	Asbestos-exposed > HC	[192]
	Asbestosis (15)	Healthy (15)	EcoScreen + EIA	Asbestosis > HC	[189]
	Asbestosis (18) Pleural plaques (26) DPT (16)	Healthy (26)	EcoScreen + EIA	Asbestosis > HC	[187]
	Asbestos-exposed (66)	Healthy, non-exposed (41)	Chemiluminescence NO analyser	Asbestos-exposed > HC	[194]
HNE	Asbestos-exposed (45)	Healthy (29)	SPE + LC-ESI-MS/MS	Asbestos-exposed > HC	[192]
8-OHdG	Asbestosis (10)	Healthy (10)	Lyophilisation + LC-ESI-MS/MS	Asbestosis > HC	[188]
FeNO	Asbestosis (12) Pleural plaques (32) DPT (12)	Healthy (35)	Chemiluminescence NO analyser	Asbestosis > HC Pleural plaques > HC	[198]
	Asbestosis (15)	Healthy (15)	Chemiluminescence NO analyser	No differences	[189]
	Asbestos-exposed (66)	Healthy, non-exposed (41)	Chemiluminescence NO analyser	Asbestos-exposed > HC	[194]
Lipid peroxidation					
Leukotriene B4	Asbestosis (45) Silicosis (37)	Healthy (27)	SPE + LC-ESI-MS/MS	Asbestosis > HC Asbestosis > Silicosis	[190]
	Asbestosis (15)	Healthy (15)	EcoScreen + EIA	Asbestosis > HC	[189]
	Asbestos-exposed (66)	Healthy, non-exposed (41)	EcoScreen + EIA	Asbestos-exposed > HC	[194]
Leukotrienes C4, D4, E4	Asbestosis (45) Silicosis (37)	Healthy (27)	SPE + LC-ESI-MS/MS	LTD4+LTE4: Asbestosis > HC Silicosis > HC LTC4: no differences	[190]

8-isoprostane = 8-*iso*-prostaglandine $F_{2\alpha}$. 8-OHdG = 8-Hydroxy-2'-Deoxyguanosin. DPT = Diffuse Pleural Thickening. EIA = Enzyme Immunoassay. FeNO = Fractional Exhaled Nitric Oxide. HC = Healthy control. HNE = 4-Hydroxy-*trans*-2-Nonenal. NA = Not Applicable.

Table 4: Volatile breath biomarkers measured with GC-MS in patients with MPM and lung cancer.

VOC	*Controls (n)*	*MPM (n)*	*Lung Cancer (n)*	*Method*	*Sample*	*Ref.*
Acetophenone	13	13		TD-GC-MS	Breath	[200]
	NA		NA	SPME + GC-TOF-MS	Urine (Mice) + *in vitro*	[201]
	31		65	SPME-GC-MS	Breath	[209]
	31		65	SPME-GC-MS	Breath	[211]
Benzene, trimethyl-	13	13		TD-GC-MS	Breath	[200]
	26 COPD/85 Healthy		36	SPME-GC-MS	Breath	[210]
	50		36	SPME-GC-MS	Breath	[203]
Cyclohexane	48 (NH)		60	TD-GC-MS	Breath	[207]
	13	13		TD-GC-MS	Breath	[200]
Cyclohexane, methyl-	13	13		TD-GC-MS	Breath	[200]
Cyclopentane	13	13		TD-GC-MS	Breath	[200]
Decane	48 (NH)		60	TD-GC-MS	Breath	[207]
	13	13		TD-GC-MS	Breath	[200]
	26 COPD/85 Healthy		36	SPME-GC-MS	Breath	[210]
	31		65	SPME-GC-MS	Breath	[211]
	13		29	SPME-GC-MS	Breath + *in vitro*	[208]
Dodecane	13	13		TD-GC-MS	Breath	[200]
	50			TD-GC-MS	Breath	[176]
	22		30	SPME-GC-MS	Breath	[204]
Heptane	13	13		TD-GC-MS	Breath	[200]
	26 COPD/85 Healthy		36	SPME-GC-MS	Breath	[210]
	31		65	SPME-GC-MS	Breath	[211]
	50		36	SPME-GC-MS	Breath	[203]
Heptane, 2,4-dimethyl-	48 (NH)		60	TD-GC-MS	Breath	[207]
	13	13		TD-GC-MS	Breath	[200]
	31		65	SPME-GC-MS	Breath	[211]
	NA		NA	TD-GC-MS	*In vitro*	[205]
1-Hexanol, 2-ethyl-	13	13		TD-GC-MS	Breath	[200]
	20		20	SPME + GC-TOF-MS	Urine	[202]

Limonene	13	13		TD-GC-MS	Breath	[200]
	50			TD-GC-MS	Breath	[176]
	31		65	SPME-GC-MS	Breath	[211]
Nonane, dimethyl-	13	13		TD-GC-MS	Breath	[200]
Octane, 3-methyl-	48 (NH)		60	TD-GC-MS	Breath	[207]
	13	13		TD-GC-MS	Breath	[200]
1,2-Pentadiene	13	13		TD-GC-MS	Breath	[200]
α-Pinene	13	13		TD-GC-MS	Breath	[200]
Styrene	48 (NH)		60	TD-GC-MS	Breath	[207]
	13	13		TD-GC-MS	Breath	[200]
	50			TD-GC-MS	Breath	[176]
	26 COPD/85 Healthy		36	SPME-GC-MS	Breath	[210]
	31		65	SPME-GC-MS	Breath	[211]
	13		29	SPME-GC-MS	Breath + *in vitro*	[208]
	50		36	SPME-GC-MS	Breath	[203]
Toluene	13	13		TD-GC-MS	Breath	[200]
	26 COPD/85 Healthy		36	SPME-GC-MS	Breath	[210]
	31		65	SPME-GC-MS	Breath	[211]
	22		30	SPME-GC-MS	Breath	[204]
	50		36	SPME-GC-MS	Breath	[203]
	67		115	SPME-GC-MS	Breath + i*n vitro*	[206]
Xylene	13	13		TD-GC-MS	Breath	[200]
	26 COPD/85 Healthy		36	SPME-GC-MS	Breath	[210]
	31		65	SPME-GC-MS	Breath	[211]
	50		36	SPME-GC-MS	Breath	[203]
	67		115	SPME-GC-MS	Breath + i*n vitro*	[206]

COPD: Chronic Obstructive Pulmonary Disease. GC-MS: Gas Chromatography-Mass Spectrometry. MCC/IMS: Multicapillary Column/Ion Mobility Spectrometry. MPM: Malignant Pleural Mesothelioma. NA: Not Applicable. NH: Non-Healthy. SPME: Solid Phase Micro Extraction. TD: Thermal Desorption. TOF: Time-of-flight. VOC: Volatile Organic Compound.

Table 5: Overview of breath analysing techniques for volatile compounds.

Gas Chromatography – Mass Spectrometry (GC-MS)	Electronic Nose (eNose)	Ion Mobility Spectrometry (IMS)	Canine Scent
▪ Sensitive (pp_m-pp_t) ▪ Identification, Detection, Quantification of VOCs ▪ Vacuum Conditions ▪ Slow ▪ *Offline* Sampling ▪ Large, Immovable Set-Up ▪ Very Expensive ▪ Specific Technician Training ▪ Gold Standard	▪ No Specific VOC Identification ▪ Blackbox ▪ Ambient Conditions ▪ Fast, Easy ▪ *Offline* Sampling ▪ Transportable ▪ Cheap ▪ No Specific Operator Training	▪ Sensitive (pp_b-pp_t) ▪ VOC Identification possible with MCC column ▪ Ambient Conditions ▪ Fast, Easy ▪ *Online* Sampling ▪ Transportable ▪ Cheap ▪ No Specific Operator Training	▪ Time Consuming (Dog Training) ▪ No Quantification/Identification of VOCs ▪ Ambient Conditions ▪ Fast, Easy ▪ *Online* Sampling ▪ Transportable ▪ Expensive ▪ No Specific Operator Training

MCC: Multicapillary Column. pp_b: parts per billion by volume. pp_m: parts per million by volume. pp_t: parts per trillion by volume. VOC: Volatile Organic Compound.

FIGURES

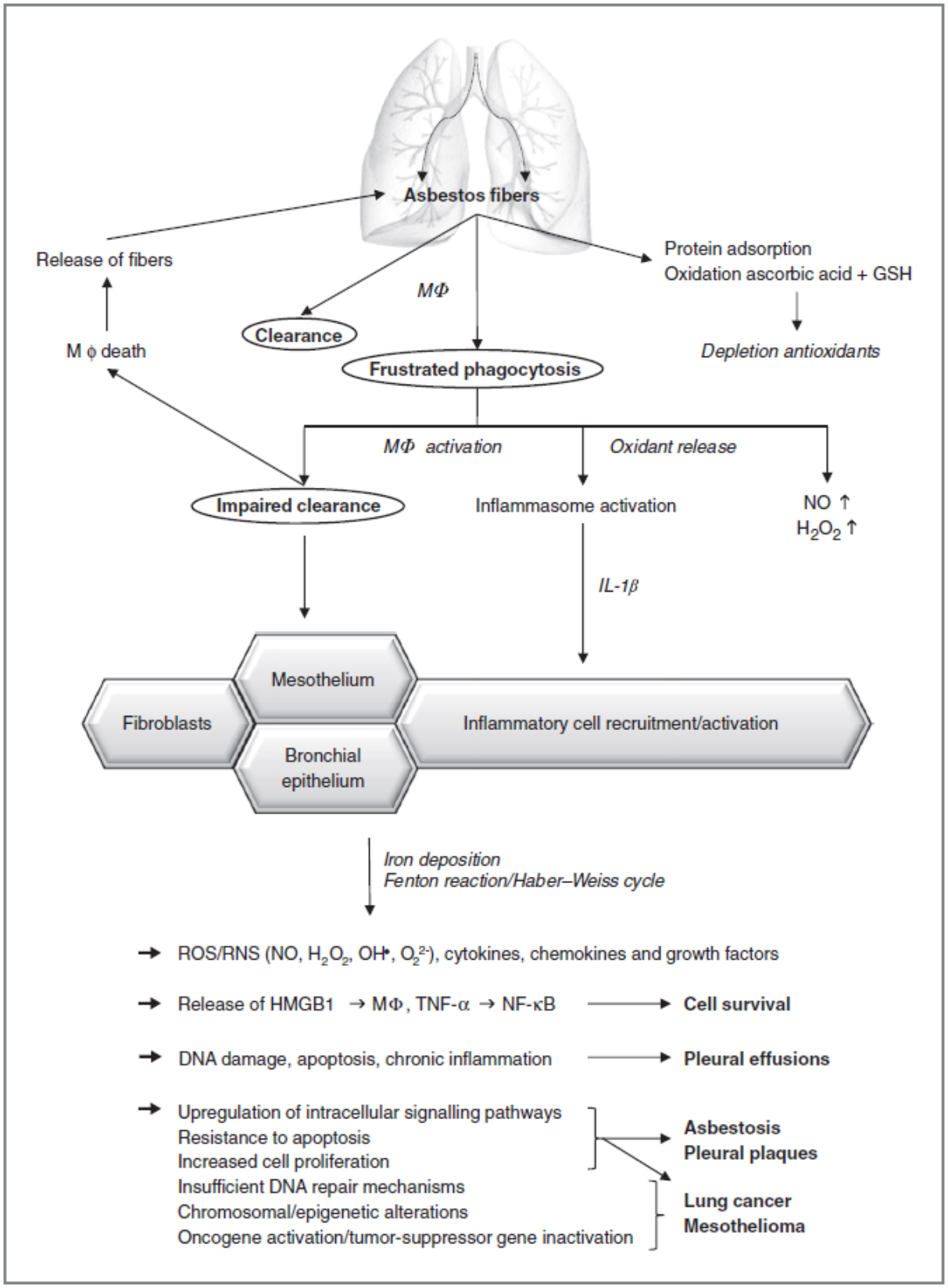

Figure 7: Pathogenesis of asbestos-related diseases. When asbestos fibres are inhaled, they penetrate the lung parenchyma and irritate the pleura. The iron content from asbestos fibres forms oxidants through the Haber-Weiss and Fenton chemistry and depletes antioxidants. Normally, fibres are cleared by macrophages but fibres which are too long are not fully engulfed by them, leading to frustrated

phagocytosis and impaired clearance of the fibres at the bronchial epithelium, mesothelium and fibroblasts. In its turn, this leads to oxidant and HMGB1 release and a chronic inflammatory response due to TNF-α release and the activation of the NF-κB pathway. Asbestos fibres also mechanically interfere with DNA leading to damage, breaks and chromosomal aberrations and induce the TNF-α receptor on mesothelial cells. Binding of TNF-α to its receptor will induce survival and proliferation of the damaged mesothelial cells and an upregulation of signalling pathways which leads to a cancerous pathogenesis. GSH: glutathione. IL-1β: interleukin 1β. MΦ: macrophages.

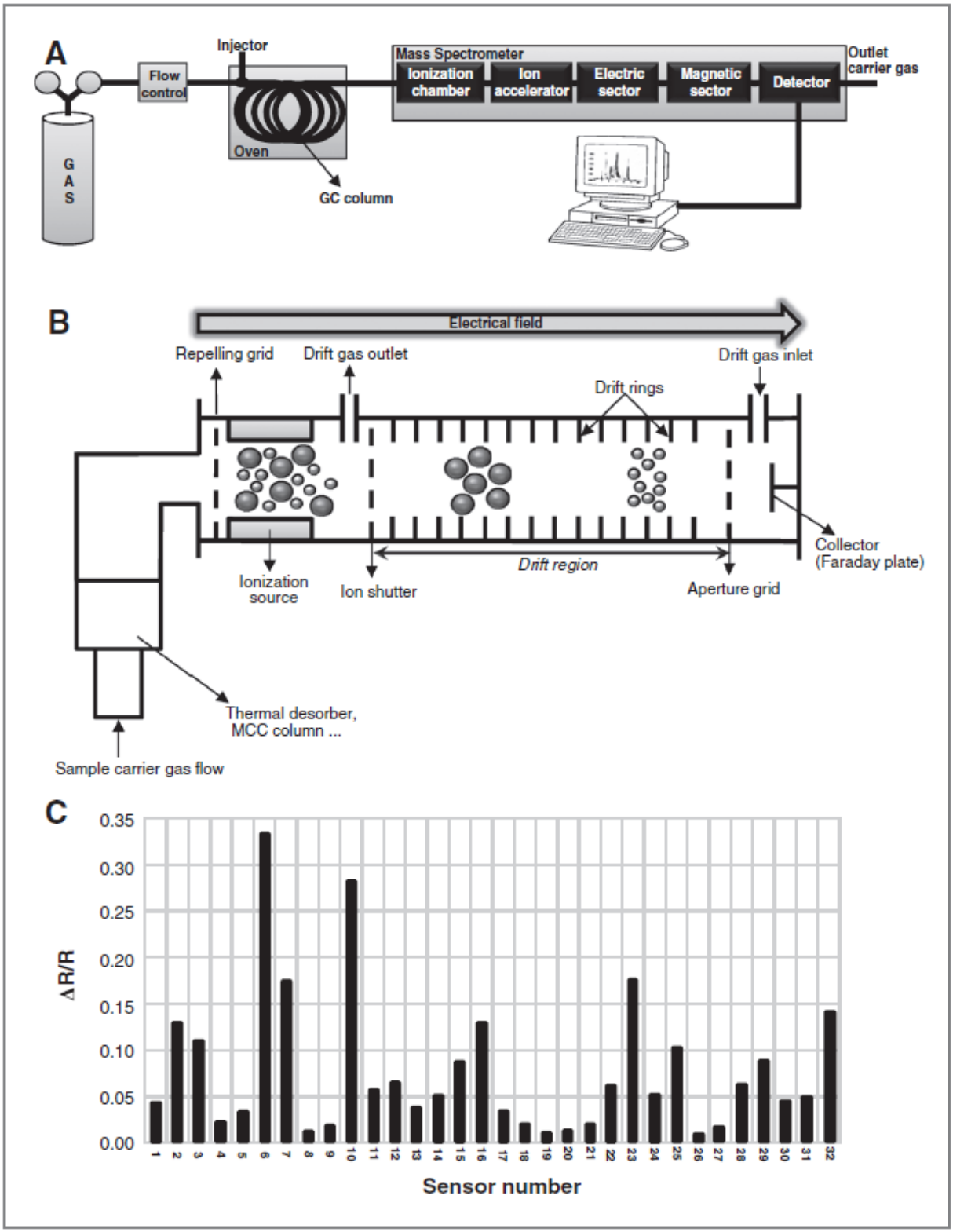

Figure 8: A. General principle of GC-MS. The breath is collected into bags (Tedlar, Mylar, Polytetrafluoroethylene) and subsequently concentrated by adsorbing them onto a cold surface (via EBC devices, cryofocussing or lyophilisation) or onto some adsorbents (Tenax columns) from which they are released into the sample inlet of the GC-MS via solid phase micro-extraction or thermal desorption. A carrier flow gas takes the sample over a heated GC column to separate the VOCs based upon their chemical characteristics. When entering the MS, samples are ionized, accelerated and led to a detector through time-of-flight or quadrupole analysis.

B. Principles of IMS. Breath samples are thermally desorbed or enter the IMS through direct sampling via a multicapillary column (MCC) to separate the different chemicals based upon their chemical characteristics and from the water content of the breath. In general, nitrogen or synthetic air is used as a carrier gas of which the molecules are directly ionized by the β-particles of a ^{63}Ni radioactive source. Positive carrier gas ions (reactant ions) and free electrons will become available and undergo different chemical reactions with the breath analyte ions to form product ions. After the ionization phase, a short opening of an ion shutter (e.g. Bradbury-Nielsen gate) every 100-300 ms for 300 µs allows the ionized breath compounds to enter a drift tube (10-15 cm) which separates the VOCs based upon their ion mobilities (charge, mass, size and shape). In the drift tube, VOCs move to a detector (e.g. Faraday plate) under the influence of a low electric field and a counter gas which generates a small electric current. The time after opening of the grid and the arrival at the detector (the drift time) is measured to characterize the ions.

C. Example of a smellprint. Subjects need to breath normally for 5 min through a three-way non-rebreathing valve with a VOC and a silica filter. After a maximal deep inspiration, subjects exhale a single vital capacity volume into a 10 L Tedlar bag connected to the expiratory port and silica reservoir, sampling all of the breath. Next, the Tedlar bag is connected to the eNose for analysis or the sample is first brought onto Tenax columns within 10 min and thermally desorbed and subsequently sampled in the eNose. The bulk of the VOCs change the resistance of the 32 polymer carbon sensors of the Cyranose 320. The change in relative resistance (ΔR/R) for 32 carbon polymer sensors is known as a smellprint.

CHAPTER 6
BIOMARKERS: FROM BENCH TO BEDSIDE

6.1 Introduction

Before a biomarker can be used in the standard-of-care diagnostic work-up, it must undergo a large number of steps, ranging from its discovery to clinical utility (Figures 9 & 10). It is essential that such tests are accurate and reliable (analytical validity; *'is it true'*), that they are associated with the disease or the outcome of interest (clinical validity, *'is it meaningful'*), and finally lead to its clinical utility (*'is it useful'*) which is defined as the evidence that the use of the test results in an improved measurable clinical outcome of value to clinical decision making compared to the routine management without the use of the test [229-233]. Clinical utility can be proven by performing clinical trials that allow improval of favourable outcomes such as survival or quality of life, improval of the clinical decision making leading to avoidance of unnecessary therapy and cost savings or that allow a decrease of the harms such as symptomatology or toxicity. These outcomes can vary with the cancer type, stage, and treatment intention [229]. The clinical utility strongly depends on the analytical and clinical validity of the biomarker and is a dynamic construct that can change over time as the evidence accumulates, clinical practice changes and new biomarkers enter the market [229, 230]. Once a biomarker have been proven useful in several clinical trials, it can be approved by the US Food and Drug Administration (FDA) for clinical use [234].

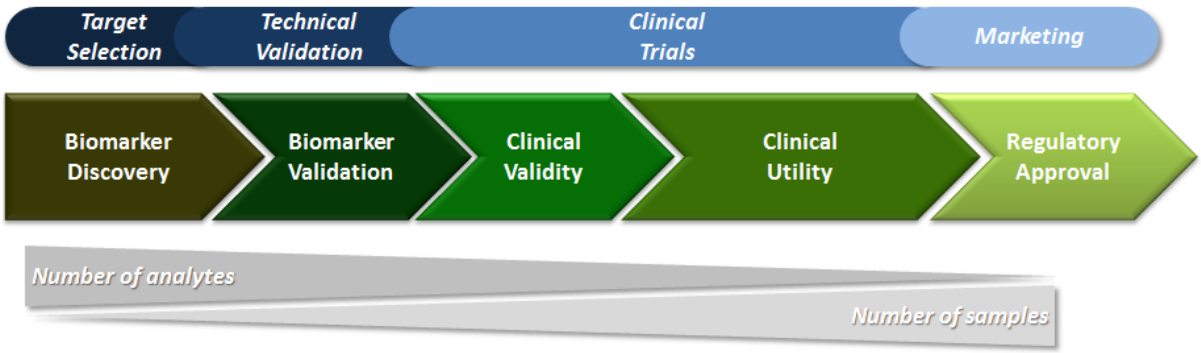

Figure 9: Biomarker development: from bench to bedside. *(Figure by Kevin Lamote)*

6.2 Steps for clinical implementation of breath biomarkers

Previous general work-flow for biomarker development can be applied to the development of breath biomarkers (Figure 10). This includes a number of steps ranging from the discovery of biomarkers to the analytical validation of the method which includes the assessment of repeatability, reliability and reproducibility, over finding biological evidence for the biomarker in its specific application, and the improvement of technology and miniaturisation [233]. Afterwards the clinical validity can be assessed and clinical utility proven by performing prospective, case-control, cohort studies before the biomarkers can be approved for clinical implementation [229, 233]. These steps will be discussed in more detail. As mentioned, biomarkers may be used to help increase the certainty of presence or absence of disease (diagnosis/screening), support clinical management, assess the prognosis or monitor treatment and, hence, the biomarker test must be evaluated in accordance with its objectives. For diagnostic or screening purposes, the accuracy of the biomarker test is an important issue and reflects the ability to distinguish between patients and controls. The sensitivity with the negative predictive value (NPV) and specificity with the positive predictive value (PPV) jointly determine the accuracy of a diagnostic test [233, 235]. When dealing with a lethal disease as mesothelioma, a test should have high sensitivity to ensure that no patients will be missed. Furthermore, when used for screening, a high NPV allows to rule out disease in patients without the disease and subsequently decrease morbidity from diagnostic delay or mistreatment. Maximizing sensitivity and NPV should be key for breathomics research regarding diagnosing mesothelioma. On the other hand, for chronic diseases like asthma, COPD or sarcoidosis, the focus lies upon specificity rather than sensitivity where one wants to correctly identify patients among the intention-to-diagnose population.

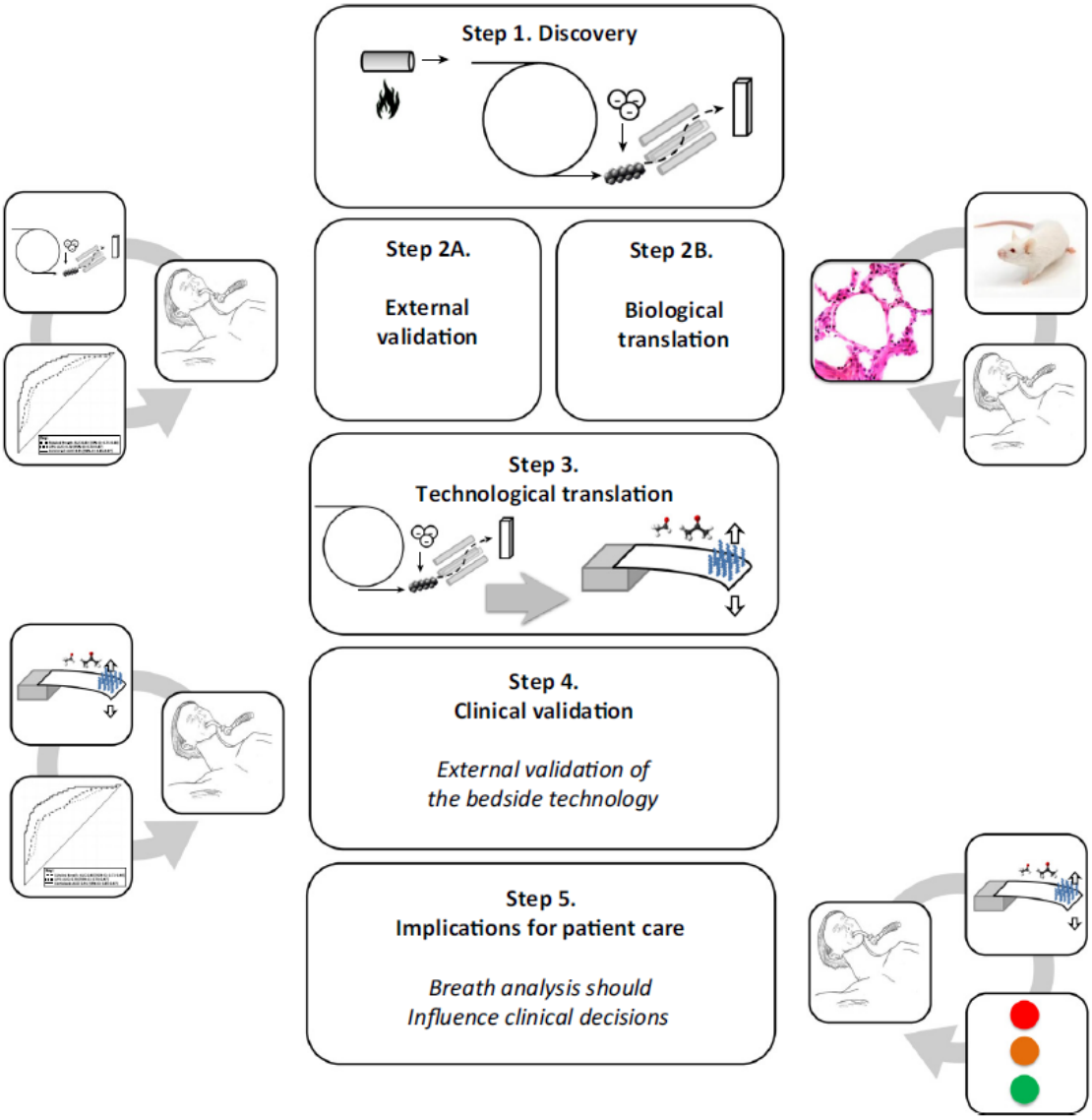

Figure 10: Steps involved for clinical implementation of a breath biomarker [159]. *Step 1 involves the discovery of VOCs indicative for disease. Step 2 involves resampling in clinical situations with analysis of the VOCs observed in Step 1 for validation purposes and indicates the search for biological evidence by analysing VOCs in cell lines, animal models, and clinical situations. Step 3 is a possible miniaturization phase where we move from a large analytical setup to small hand-held devices. Step 4 depicts the same process as step 2, a clinical validation, using the hand-held device instead of the gold standard. Step 5 states that, ultimately, breath analysis should be used to make a decision in clinical situations with respect to either diagnosis or treatment.*

6.2.1 Discovery

Chemical identification of VOCs aims to discover pathophysiological mechanisms related to disease. The current gold standard within breathomics to perform breath analysis is GC-MS. This technique allows to separate VOCs based upon their specific interaction with the stationary phase of the applied chromatography column, after which they are ionized and

identified based on their mass-to-charge (m/z) ratio [236, 237] (Chapter 5, page 52). Since these characteristics are specific for every VOC, identification becomes possible by comparison with a mass spectral library. This allows to theoretically identify every VOC and gain insights in the underlying biological mechanisms [238]. As already discussed, this technique is time-consuming, and requires special technical issues, limiting its direct clinical use. Next to GC-MS, MCC-IMS can also be used. It contains a multicapillary (MCC) column which allows a preseparation of VOCs based upon their interaction with the stationary phases of the MCC, generating a specific retention time to traverse the MCC. Afterwards, the VOCs are ionized and separated based upon their ion mobility characteristics [239]. The combination of both characteristics are specific for every VOC and hence, again a theoretical identification is possible by comparison with the ion mobility libraries. Next to these technical issues, unsupervised and supervised statistical analyses are required for selecting discriminatory VOCs in relation to the disease but one has to be careful for the risk of overfitting and false discoveries [240]. In order to identify interesting compounds, the first step is to perform cross-sectional studies wherein the patients and controls are clearly characterized so no heterogeneity would occur that could impede biomarker discovery. Although this induces a highly selection of patients and controls, it will ultimately allow us to seek for those key compounds that differ between the two extremes [235].

6.2.2 Internal validation

Before biologically and externally validating the findings, the methods for breath analysis have to demonstrate an adequate reproducibility and transferability. After potential discriminatory compounds have been identified, the results have to be repeated [159] in order to assess the analytical validity. This is done preferably by repeating the study, including more patients and following the same protocol or by using a test set and validation set or by performing k-fold cross-validation.

6.2.3 Biological translation

As stated in Koch's postulates, a biomarker should be linked to the disease of interest. Despite progress over the past years, the biochemical origin of most VOCs is still largely unknown [159] and underlines the importance to understand the biochemical pathways that give rise to the VOCs and link the VOCs with the biological presence of disease. Although intuitively the biological mechanisms that induce VOCs in breath are not of primary interest for diagnostic purposes, VOC identification is of key importance in the initial evaluation of a diagnostic problem because the identity of the VOCs can aid to develop more targeted disease-specific sensor-based bedside tests in future phases of biomarker development [241].

In order to elucidate biological pathways that link VOCs to a specific disease, a translational approach is advocated, including analysis of *in vitro* cell cultures and/or experimental animal studies and should focus on explaining the variance instead of diagnosing the disease. *In vitro* studies will help to understand the origin and kinetics of VOCs and expanding research to animal models will help gain insights related to the VOC metabolism *in vivo*. This translational bridge is necessary since it will aid in determining the clinical validity and improve the diagnostic accuracy once the factors influencing the biological levels are known.

6.2.4 Technology Miniaturisation

When clinical application at regular point-of-care is required, the optimal approach is to create a hand-held, easy-to-use, device for physicians by either miniaturizing GC-MS devices or by developing small hand-held sensors arrays capable of detecting sets of the identified discriminating VOCs.

6.2.5 Clinical validation

The validation of the diagnostic performance of a biomarker in human clinical trials is crucial. Therefore, the diagnostic performance of the miniaturised diagnostic device should be tested again, preferably in a blinded, prospective, case-control cohort study, following persons at risk over time and to assess the clinical utility and the final place in the diagnostic work-up for

MPM. There are guidelines regarding the analytical steps required in the design of such trials for biomarker validation in clinical diagnosis [242] following the recommendations for diagnostic studies [243, 244].

6.2.6 Regulatory approval for clinical use

After the biomarker test has have been proven clinically useful, it can claim regulatory approval, for instance from the FDA, after complying with a stringent set of rules and regulations before getting entered on the market [234, 245]. The specific approval process will depend on the classification of the breath test which in turn is based upon its risks and the complexity of the technology. Depending on the level of regulatory control that is necessary to assure safety and effectiveness, breath tests can be classified into different device classes [234]. Class I devices induce the least risk to public health and are subject to the least regulatory control. Class II devices have moderate risk and include any device for which there is a reasonable guarantee of safety and effectiveness. Examples of class II devices are biomarkers for prognosis and monitoring in patients already diagnosed with the disease. Most class I or II devices that are not exempt from pre-market review requirements are 'cleared' for commercial distribution by the 510(k) process, taking about 100 days to be reviewed and costs about $5,228 on user fees [246]. This requires a manufacturer to demonstrate that a new product is as safe and as effective or equivalent to a product that is already being legally marketed. Traditionally, new products that are not similar to products already on the market are automatically classified as Class III, the most stringent regulatory category with the most risk to public health. These devices require the Pre-Market Approval (PMA) process which takes about 180 days to 1 year and costs $261,388 on user fees [246]. Although Class III devices include life sustaining devices and other devices that pose a potential direct risk to the patient, new *in vitro* devices (blood tests) are included in Class III since use of the device could result in harm if it leads to inappropriate interventions, for instance markers for diagnosing or screening for cancer. Since breath analysis will be used for screening, and help enrich people at risk for future monitoring, it is expected to be classified as class III.

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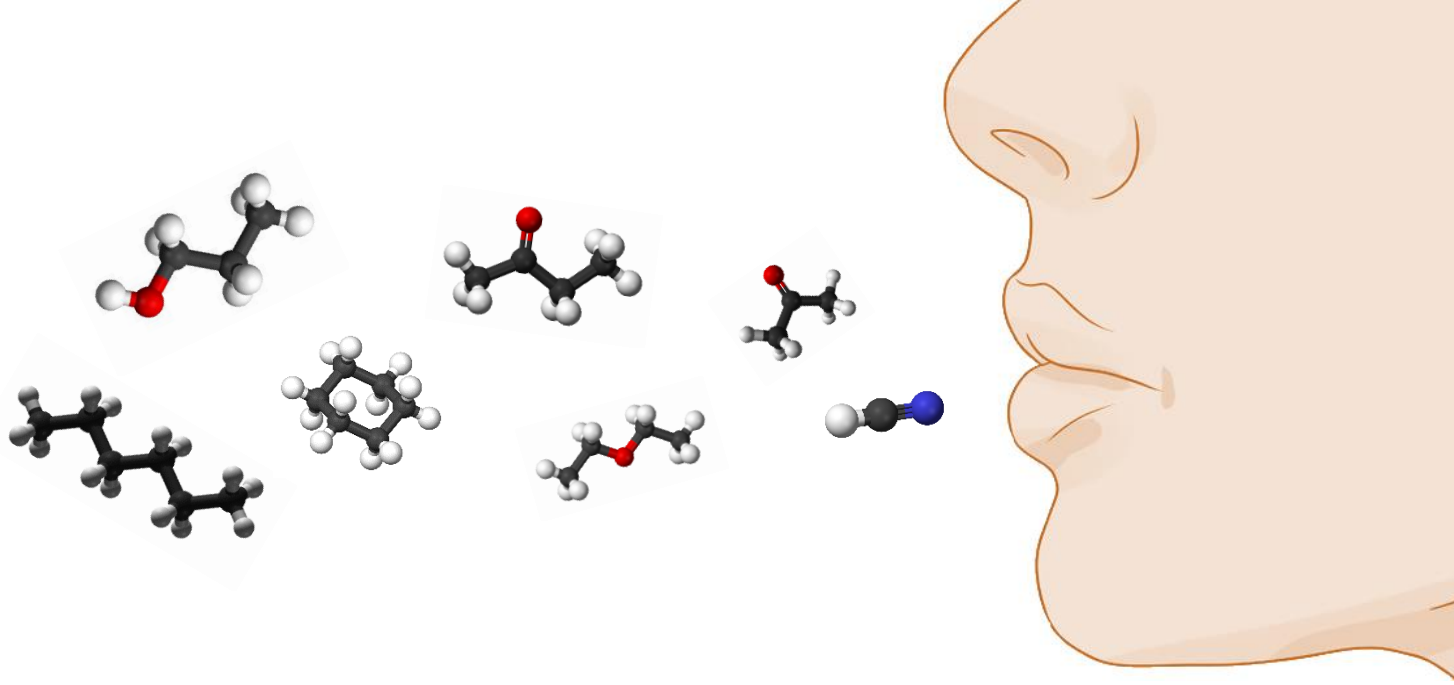


PART II:

RESEARCH AIMS

AIMS OF THE THESIS

This research works aims to explore the role of breath analysis as screening and diagnostic tool for malignant pleural mesothelioma. Therefore, we aimed to provide the first steps in breath biomarker development and used three different breath sampling methods: multicapillary column/ion mobility spectrometry (MCC/IMS), gas chromatography – mass spectrometry (GC-MS) and sensor technology (electronic nose; eNose).

Although the incidence of MPM is increasing, it still remains a rare disease with Belgium having only 273 new incident cases in 2013. In order to obtain a sufficient number of patients and participants, we initiated a cross-sectional, case-control series (called the MesoBreath series), wherein a collaboration with the departments of Respiratory Medicine of the University Hospitals of Ghent, Leuven and Antwerp was established. Furthermore, we collaborated with a company that had used asbestos in their products until 1997 in order to include a representative control population of asymptomatic persons at risk for MPM.

In total, we aim to include 300 participants, divided over six different patient and control groups, in a timespan of three years (Table 4). These include (a) healthy controls without known historical occupational asbestos-exposure, (b) asymptomatic persons with a known historical occupational asbestos exposure, (c) patients with benign asbestos-related diseases (Part I, Chapter 2), (d) patients with benign non-asbestos related respiratory diseases, (e) primary lung cancer patients and (f) mesothelioma patients who were all treatment-naïve.

Table 4: Participant groups of the MesoBreath series

Group	Aim (N)	Included (N)
Healthy controls	50	52
Healthy asymptomatic asbestos-exposed individuals	50	59
Patients with benign asbestos-related diseases	50	41
Patients with benign non-asbestos related respiratory diseases	50	70
Lung cancer patients	50	56
Mesothelioma patients	50	52
TOTAL	**300**	**330**

In **Part III – Chapter 1**, we aim to establish a good sampling protocol that allows to discriminate mesothelioma patients from asymptomatic controls with historical asbestos exposure (AEx) and without this exposure (HC) with an acceptable accuracy. Therefore, a multicentre, cross-sectional study was initiated including a limited number of patients and controls. By doing so, we initiate the first step in biomarker development and explore the analytical validity (Figure 11). Furthermore, we want to discover important volatile breath biomarkers that allowed the discrimination.

In **Part III – Chapter 2**, we aim to confirm the analytical validity by repeating the study using the same protocol and including more patients and different patient groups (Figure 11). Together with the previous study, this allows us to pinpoint interesting discriminatory VOCs and to assess the discriminatory capacity to differentiate mesothelioma patients from confounding groups.

In **Part III – Chapter 3**, we want to technically validate the findings by comparing the breath sampling method with the gold standard, GC-MS. By performing GC-MS analysis in several patients and controls, we will ultimately be able to identify discriminatory VOCs. Furthermore, in preparation for the miniaturisation phase and to investigate the possibility to detect MPM with hand-held sensor technology, we validated the GC-MS findings by performing parallel eNose analysis (Figure 11).

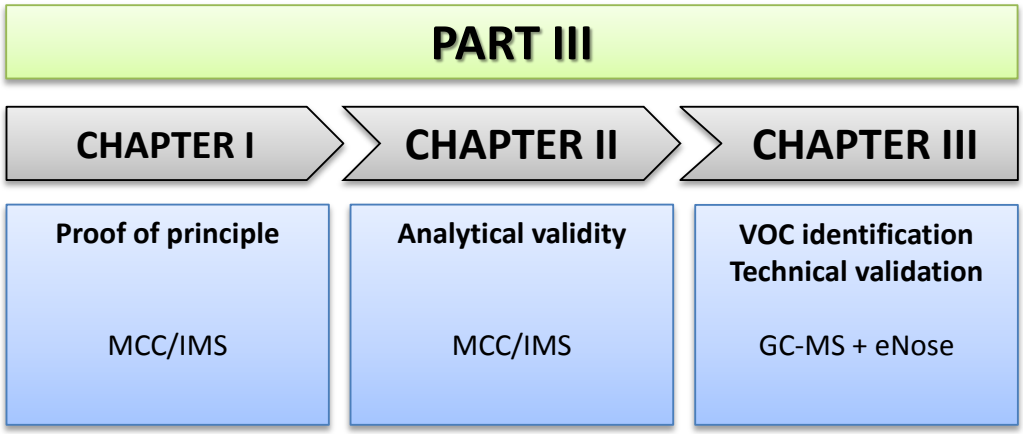
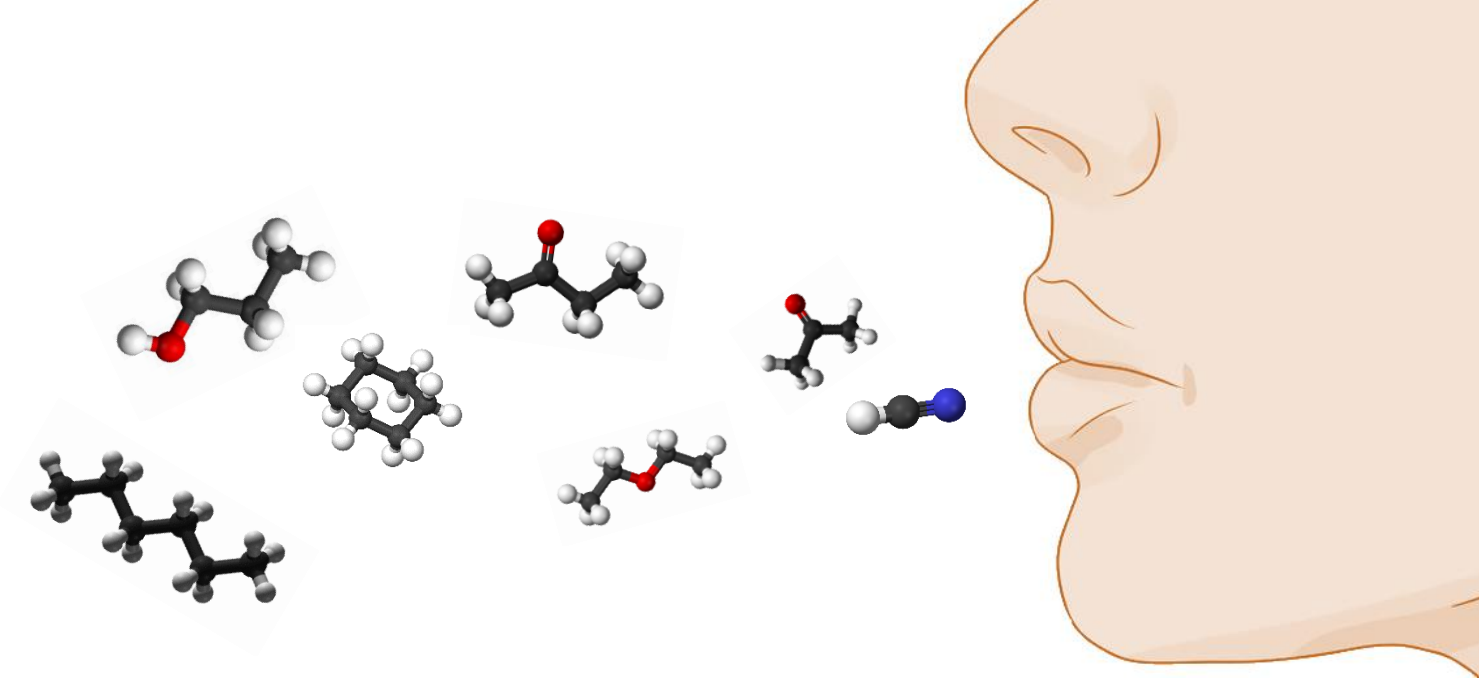


Figure 11: Overview of the research aims. MCC/IMS: multicapillary column/ion mobility spectrometry. GC-MS: gas chromatography-mass spectrometry. eNose: electronic nose.

In summary, this thesis aims to fulfil the following goals:

1) Set up a cross-sectional, case-control series of studies (the MesoBreath series) to evaluate the role of VOCs in screening for MPM and its diagnosis.
2) To determine the diagnostic and screening value in discriminating MPM from at risk control groups.
3) To identify discriminatory compounds and determine the analytical validity of the biomarker test.
4) To validate the findings by using essentially different technologies for molecular assessment in exhaled breath.



PART III:

SCIENTIFIC RESEARCH

CHAPTER 1

Detection of malignant pleural mesothelioma in exhaled breath by multicapillary column/ion mobility spectrometry (MCC/IMS).

Lamote K, Vynck M, Van Cleemput J, Thas O, Nackaerts K, van Meerbeeck JP. *Journal of Breath Research.* 2016:10(4):046001.

ABSTRACT

Background: Malignant Pleural Mesothelioma (MPM) is predominantly caused by previous asbestos exposure. Diagnosis often happens in advanced stages restricting any therapeutic perspectives. Early stage detection via breath analysis was explored using multicapillary column/ion mobility spectrometry (MCC/IMS) to detect volatile organic compounds (VOCs) in the exhaled breath of MPM patients in comparison to former occupational asbestos-exposed and non-exposed controls.

Methods: Breath and background samples of 23 MPM patients, 22 asymptomatic former asbestos (AEx) workers and 21 healthy non-asbestos exposed persons were taken for analysis. After background correction, we performed a logistic least absolute shrinkage and selection operator (lasso) regression to select the most important VOCs, followed by receiver operating characteristic (ROC) analysis.

Results: MPM patients were discriminated from both controls with 87% sensitivity, 70% specificity and respective positive and negative predictive values of 61% and 91%. The overall accuracy was 76% and the area under the ROC-curve was 0.81. AEx individuals could be discriminated from MPM patients with 87% sensitivity, 86% specificity and respective positive and negative predictive values of 87% and 86%. The overall accuracy was 87% with an area under the ROC-curve of 0.86.

Conclusion: Breath analysis by MCC/IMS allows MPM patients to be discriminated from controls and holds promise for further investigation as a screening tool for former asbestos-exposed persons at risk of developing MPM.

Belgian registration number B670201111954

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a tumour predominantly caused by asbestos exposure [1, 2]. Most West-European countries banned the use of asbestos in the 1990s and regulations banning the use of asbestos became effective in the entire European Union in 2005. However, the continued asbestos usage in low-income regions and communities, Russia, China and India remains worrisome. Most countries faced increasing numbers of occupational exposures up to the 1970s. In studies with adequately long follow-up, an average latency of 40-50 years since first exposure [3, 4] explains the observed increase in mesothelioma incidence and why substantial decreases are not expected before 2025.

A formal tissue diagnosis obtained by thoracoscopy is essential for MPM management [2]. With a median survival of less than one year and a five-year survival rate below 5%, prognosis is poor [1]. Therefore, research has focused on early detection by using several blood biomarkers. Despite promising trials, soluble mesothelin-related protein, osteopontin and megakaryocyte potentiating factor were not suitable as early detection tools [5, 6], whereas fibulin-3 and connective tissue growth factor need further prospective validation [7, 8], urging the need for other non-invasive screening biomarkers.

Recently, breath analysis emerged as a high-throughput *breathomics* research field [9]. Exhaled breath is easily accessible without patient discomfort and contains trace elements known as volatile organic compounds (VOCs) [10-13] which arise from the (patho)physiological processes in the body. For instance, acetone is linked to lipolysis via decarboxylation of excess acetyl-CoA, the mevalonic pathway in cholesterol synthesis liberates isoprene and lipid peroxidation of (poly)unsaturated fatty acids by oxidative stress in cellular membranes induces the formation of several hydrocarbons [11, 13, 14]. These VOCs enter the bloodstream, are transported to the lungs where they enter the alveoli through gas exchange mechanisms and are subsequently released in the exhaled breath. Until today, de Gennaro *et al.* found cyclohexane to discriminate breath samples of 13 MPM patients, 13 occupational asbestos-exposed persons and 13 healthy non-exposed controls using gas chromatography-mass spectrometry (GC-MS) with 97.4% accuracy [15]. Furthermore, Dragonieri *et al.* [16] used an electronic nose (e-Nose) to compare breath samples of the same three groups and distinguished MPM patients from controls with 92.3% sensitivity. Chapman *et al.* repeated this e-Nose study and distinguished MPM patients with 90% sensitivity and 88% specificity [17]. Since asbestos causes oxidative stress of the mesothelium [18, 19],

tumours upregulate their metabolism and these small-sized studies suggest that MPM patients can be distinguished from controls based upon breath patterns [16, 17]; we hypothesize that the VOC composition in the breath of MPM patients differs from those in the breath of controls and that breath analysis can be used to screen for MPM in an at risk population.

MATERIALS AND METHODS

Design, participants and settings

This is a multicentre, cross-sectional, case-control study. MPM patients and healthy non-asbestos exposed controls (HC) were recruited from the University Hospitals of Ghent, Leuven and Antwerp (Belgium). Asymptomatic former asbestos workers (AEx) were recruited via the occupational health service of a fibre-cement factory that used asbestos until 1997. MPM diagnosis was verified by the Belgian Mesothelioma Pathology Panel. Exclusion criteria were (1) the start of any anti-tumour treatment before breath sampling in MPM patients and (2) the presence of other asbestos-related diseases in the control groups (a recent CT-scan or chest X-ray <12 months was mandatory to confirm the medical condition). Participants had to refrain from eating, drinking and smoking at least two hours before breath sampling. Two questionnaires were taken at inclusion: one to check whether participants met the inclusion criteria and one to collect demographical and past occupational asbestos exposure data. A detailed patient record with all details of the patient's medical condition was available.

The study was approved by the Institutional Review Board of Ghent University Hospital (LONG 11-01; Belgian registration number B670201111954) and was conducted according to the Helsinki Convention. Participants had to give their written informed consent before inclusion.

Technical information ion mobility spectrometer (IMS)

A BioScout breath analysing device was used operating on VOCan v2.4 software (B&S Analytik, Dortmund, Germany). It consists of a BreathDiscovery ion mobility spectrometer (IMS) coupled to a multicapillary column (MCC), which is connected to a SpiroScout ultrasound-controlled breath sampler (Ganshorn Medizin Electronic, Niederlauer, Germany) by a sample loop. By capno-volumetry, the SpiroScout detects the CO_2-levels in exhaled breath and starts the breath sampling when a plateau in CO_2-levels is reached, indicating alveolar air is sampled. Table 1 summarizes the MCC/IMS characteristics and its principles are previously described [13, 20].

Table 1: Characteristics of the BreathDiscovery ion mobility spectrometer.

Parameter	
Ionisation source	^{63}Ni (95 MBq)
Electrical field strength	320 V/cm
Length of the drift region	12 cm
Diameter of the drift region	15 mm
Length of the ionisation chamber	15 mm
Shutter opening time	300 µs
Shutter impulse time	100 ms
Drift gas	α_1-nitrogen gas (99.999% pure, CAS-n°: 7727-37-9)
Drift gas flow	100 ml/min
Carrier gas flow	100 ml/min
Working temperature	Ambient temperature
Pressure	Ambient pressure (101 kPa)
MCC	OV-5, non-polar, 1000 packed columns, 3 mm diameter, 20 cm length
Column temperature	40°C, isothermal, adjusted
Polarity	Positive mode

Briefly, the MCC/IMS uses a 95MBq ^{63}Ni β-radiation source for the ionisation of the carrier gas (α_1-nitrogen gas, Air Liquide Medical, 99.999% pure, CAS-n°: 7727-37-9, Schelle, Belgium). After breath sampling, the gaseous breath analytes enter a non-polar OV-5 MCC column (Multichrom Ltd, Novosibirsk, Russia) for pre-separation based upon the analytes' chemical characteristics. After passing the MCC column, the pre-separated volatiles enter the ionization chamber of the IMS with a certain retention time. In this ionization chamber, the breath volatiles become positively charged through secondary ionization and charge transfer reactions by colliding with the ionized carrier gas. Subsequently, the ionized breath compounds enter a drift tube where a second separation takes place based upon their ion mobility characteristics (size, charge, mass and shape) under the influence of an electrical field and a counter gas. Finally, the VOCs collide on a Faraday plate detector where they evoke an electrical current, which results in a VOC peak intensity (Volt, V) which correlates to its concentration. The drift time from entering the drift tube until collision at the Faraday detector is also measured.

Exhaled breath sampling

Breath samples were taken between January, 1st 2012 and December, 20th 2014. All participants were asked to first rinse their mouth with distilled water and put on rubber gloves and a nose clip. Next, while sitting in an upright position at rest and without performing forced

breathing manoeuvres, they were asked to breathe calmly through the SpiroScout's mouthpiece, connected to a bacteria filter and the MCC/IMS sample loop. After three minutes of calm breathing, 10 ml of alveolar air was sampled and sent to the MCC/IMS for immediate analysis. After breath analysis, 10 ml of ambient air was sampled as background reference for every participant using an internal pump. In order to rule out external contamination or sampling artefacts as much as possible, we used disposable mouthpieces and filters. Furthermore, the unheated sample lines are made of inert Teflon (PTFE) [21], and in between the breath sampling of different participants, the MCC/IMS was flushed at least 10 times with humid air to remove contaminants and to make sure that the MCC/IMS spectra were clean.

Statistics

VOC analysis was done with VisualNow v3.7 software (B&S Analytik, Dortmund, Germany). The raw data consists of 2D-chromatograms with individual VOCs separated by retention time (s; from the MCC column) on the y-axis and their inverse reduced ion mobility (Vs/cm^2; linked to the drift time in the IMS) on the x-axis (Figure 1(a)). Next, the chromatograms were pre-processed (Figure 1(b)) by aligning all chromatograms and denoising through baseline correction using a 5x3 low pass filter (Figure S1(b)) [22]. The data was subsequently normalized to the reactant ion peak (RIP), which is the output from the ionized carrier gas, by estimating its shape and subtracting it from the measured spectra. (Figure S1(c)) and compensated for RIP-tailing by subtracting a median spectrum form each spectrum within the data set [22] (Figure S1(d)). Next, the data was smoothed (Figure S1(e)). After a visual inspection of all breath and background samples, 89 VOCs were manually selected (Figure 1(c)) and subsequently analysed resulting in a list of VOC-peak intensities (maximum peak height in the selected peak area) (Figure 1(d)).

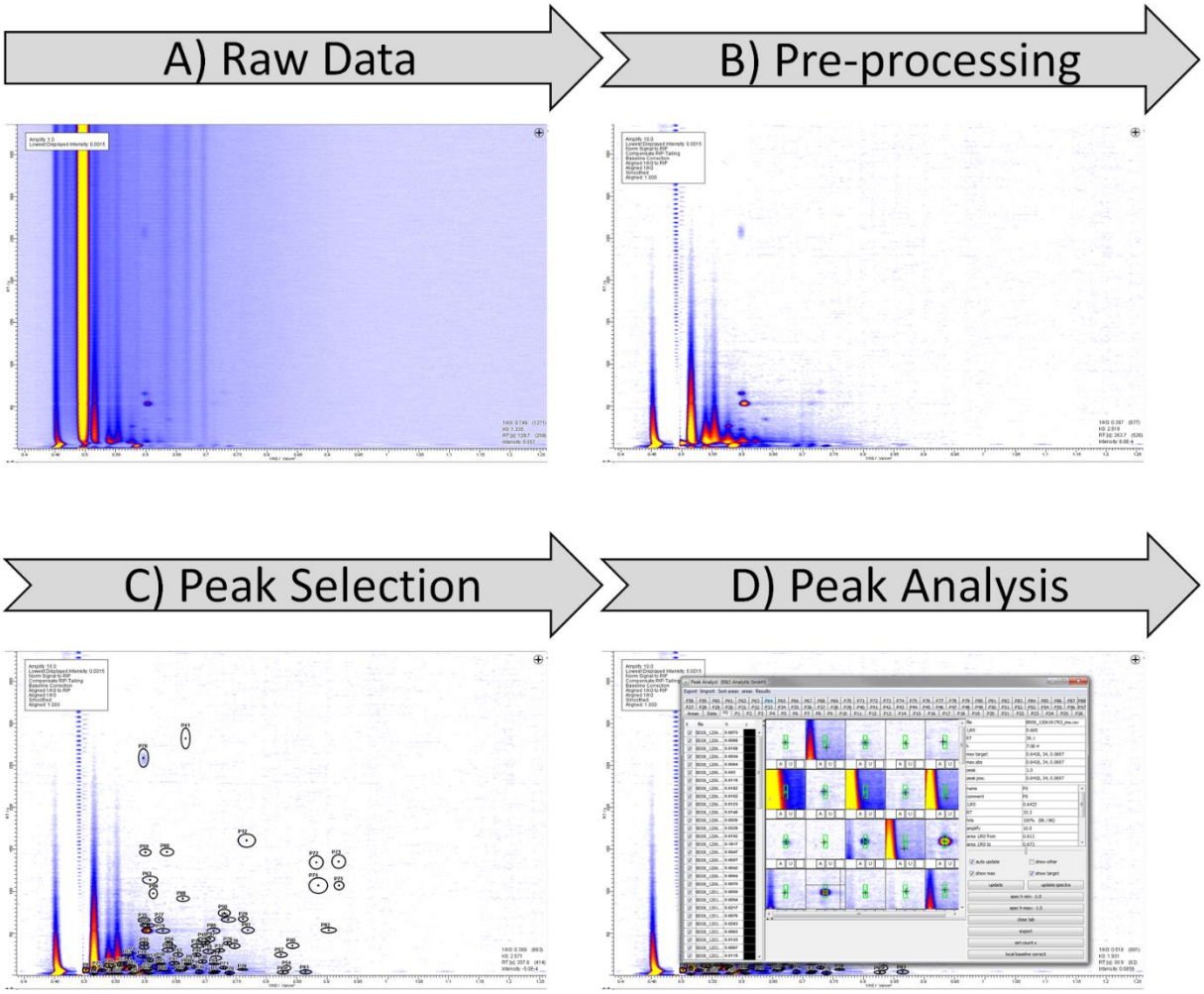


Figure 1: Data analysis scheme. **A:** Raw data. **B:** Data after pre-processing by denoising and smoothening. **C:** Manual selection of 89 volatile compounds in breath and background samples. **D:** Optimization of peak selection and analysis of the maximum peak intensity.

To remove the impact of environmental chemical confounders, the alveolar gradient was calculated for every VOC by subtracting the standardized peak intensity in the background samples from the standardized peak intensity in the corresponding breath samples [23]. These alveolar gradient intensities (Volt, V) were then processed as predictors together with the patient characteristics and clinical data using R software (version 3.2.4) [24].

The high number of variables and the rather low number of samples requires an approach like penalized logistic regression using the least absolute shrinkage and selection operator (lasso) to search for peaks that have the most discriminative power for distinguishing MPM patients from asymptomatic former asbestos workers (AEx) and healthy controls (HC). We used the *glmnet* R-package (version 2.0-2) for fitting a binomial lasso logistic model [25]. Advantages of this approach include the ease of model interpretability and variable selection (i.e. removal of non-informative peaks). Fitting these models involves the selection of a tuning parameter (λ)

that determines the number of selected peaks. The optimal λ is selected by fitting the model for a sequence of λ-values, and for each of the λ-values the fitted model is evaluated by estimating the misclassification error rate by leave-one-out cross-validation (LOOCV). The λ-value minimizing this error rate was selected and used to fit the final model. Using the predicted outcomes of all of the patients, we then constructed an ROC curve (using the *ROCR* R-package (version 1.0-7) [26]) and estimated sensitivity, specificity, positive (PPV) and negative predictive value (NPV) and the diagnostic accuracy of the final model and their 95% confidence intervals. We furthermore had a look at the number of times (the number of folds) a VOC was selected by the lasso regressions. Variables selected in a large proportion of folds (>50%) were considered important. We performed binary lasso regressions using the same approach to compare MPM patients to HC controls, MPM patients to AEx controls and AEx to HC controls. For discriminating MPM patients from pooled AEx and HC controls with only gender and smoking status as predictors, a generalized linear model was fitted, again using LOOCV to assess classifier performance.

Summary statistics of the continuous variables were calculated. A chi-squared test or Fisher's exact test was used to test whether the categorical outcomes were equally likely. For continuous variables, a Kolmogorov-Smirnov test was performed to assess normality and subsequently a t-test or analysis of variance (ANOVA) was performed to assess differences of means for the normally distributed variables. For continuous variables showing deviation from normality, differences in their distribution were assessed using the Wilcoxon-Mann-Whitney test or Kruskal-Wallis test. A significance level of 5% was used throughout the analyses.

RESULTS

Patient characteristics

Sixty-six participants were included from which a breath sample was taken: 23 treatment-naïve MPM patients, 22 AEx persons and 21 HC individuals, all without comorbidities (Table 2).

Table 2: Patient characteristics.

	MPM	AEx	HC	*p-value*
N	23	22	21	
% males	91	96	91	*0.863*[a]
Age (years)[#]	66 (59 – 73)	56 (55 – 57)	56 (40 – 58)	*<0.001*[b]
BMI (kg/m²)[§]	24.31 (2.69)	26.76 (3.09)	25.35 (3.37)	*0.032*[c]
Smoke status (never/current/ex)	9/5/9	5/5/12	13/0/8	*0.032*[a]
Pack years[#,Δ]	2.0 (0 – 26)	7.0 (1 – 22)	0 (0 – 2)	*0.009*[b]
% occupational asbestos exposure	61	100	0	*<0.001*[a]

[a]Fisher's Exact-test.
[b]Kruskal-Wallis test.
[c]One-way ANOVA test.
[#]Median (Q1 – Q3).
[§]Mean (SD).
[Δ]Self-reported.
AEx: Asymptomatic former asbestos workers. HC: Healthy non-asbestos exposed control persons. MPM: Malignant pleural mesothelioma patients.

MPM patients were significantly older (median 66 years) than AEx persons (median 56 years; p-value<0.001) and HC controls (median 56 years; p-value<0.001). There were more current and ex-smokers in the MPM (14/23) and AEx (17/22) group compared to healthy controls (8/21) and MPM patients had a lower BMI (mean 24.3 kg/m²) compared to AEx persons (mean 26.8 kg/m²; p-value=0.027). All AEx and none of the HC persons had an occupational asbestos exposure while 61% of MPM patients had occupational asbestos exposure.

Modelling

Using LOOCV for discriminating MPM patients from pooled AEx and HC controls, 66 models were evaluated.

With only gender and smoking status as clinical variables in the model selection, ROC analysis showed a diagnostic accuracy of 62% (50%-73%) with a sensitivity of 17% (6%-37%), a specificity of 86% (73%-94%) and a respective PPV and NPV of 40% (14%-71%) and 66% (53%-

78%). The area under the ROC-curve (AUC_{ROC}) was 0.17 (0.04-0.32) (Table 3, Figure 2(a)). Although we expect a classifier with no predictive quality to have an AUC of 0.5, this may be negatively biased in small samples resulting in an AUC<0.3 [27]. Gender and smoking status were close to balanced across the outcome groups, thus little predictive quality can be expected.

When allowing the 89 VOCs in the lasso procedure, the final selected model improved to 87% sensitivity (69%-97%), 61% (43%-76%) PPV and 91% (77%-98%) NPV, with a specificity of 70% (55%-82%). The overall accuracy was 76% (64%-98%) in differentiating MPM patients from AEx and HC controls with an AUC_{ROC} of 0.81 (0.69-0.91) (Table 3, Figure 2(a)). For this discrimination, the VOCs P3, P5, P50 and P71 were selected most often (Table 4).

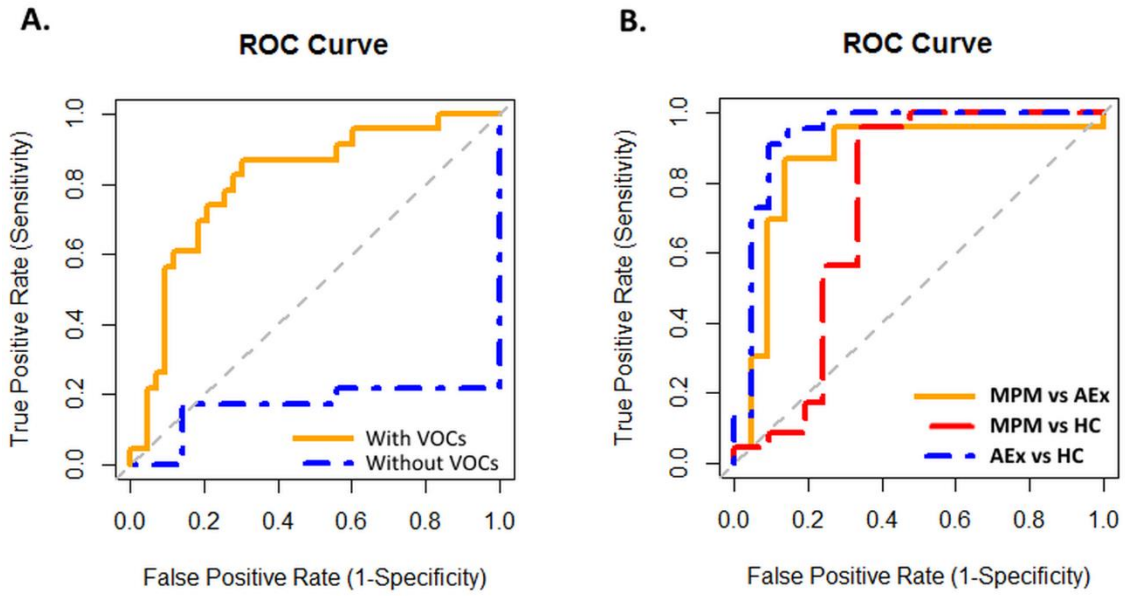


Figure 2: ROC-analysis. **A:** Model comparing the effect of clinical variables and VOCs in differentiating MPM patients from pooled AEx and HC controls (solid (orange) line = with VOCs; dotdash (blue) line = clinical variables without VOCs). **B:** Model comparing the groups separately (dotdash (blue) line = AEx vs. HC; solid (orange) line = MPM vs. AEx; dash (red) line = MPM vs. HC). The diagonal dashed line represents an uninformative test corresponding to a random chance diagnosis. AEx: asymptomatic former asbestos workers. HC: healthy controls. MPM: malignant pleural mesothelioma patients.

Table 3: Model characteristics.

	Clinical characteristics[b]	Clinical characteristics[b] + VOCs			
Model characteristics[a]	MPM vs. HC+AEx	MPM vs. HC+AEx	MPM vs. AEx	AEx vs. HC	MPM vs. HC
Sensitivity	0.17 (0.06 – 0.37)	0.87 (0.69 – 0.97)	0.87 (0.69 – 0.97)	0.95 (0.80 – 1.00)	0.96 (0.80 – 1.00)
Specificity	0.86 (0.73 – 0.94)	0.70 (0.55 – 0.82)	0.86 (0.67 – 0.96)	0.86 (0.66 – 0.96)	0.67 (0.45 – 0.84)
PPV	0.40 (0.14 – 0.71)	0.61 (0.43 – 0.76)	0.87 (0.69 – 0.97)	0.88 (0.70 – 0.97)	0.76 (0.58 – 0.89)
NPV	0.66 (0.53 – 0.78)	0.91 (0.77 – 0.98)	0.86 (0.67 – 0.96)	0.95 (0.77 – 1.00)	0.93 (0.71 – 1.00)
Accuracy	0.62 (0.50 – 0.73)	0.76 (0.64 – 0.85)	0.87 (0.74 – 0.94)	0.91 (0.79 – 0.97)	0.82 (0.68 – 0.91)
AUC_{ROC}	0.17 (0.04 – 0.32)	0.81 (0.69 – 0.91)	0.86 (0.73 – 0.97)	0.94 (0.84 – 1.00)	0.73 (0.55 – 0.89)

[a]with 95%CI.

[b]gender + smoking status.

AEx: Asymptomatic former asbestos workers. AUC_{ROC}: Area under the receiver operator characteristics curve. HC: Healthy non-asbestos exposed control persons. MPM: Malignant pleural mesothelioma patients. NPV: Negative predictive value. PPV: Positive predictive value.

Table 4: VOC characteristics.

Peak	RT (s)	$1/K_0$ (V/cm^2)	Times selected (%) and direction				Alveolar gradient (V)[a]			*p-value*[b]
			MPM vs. AEx+HC	MPM vs. AEx	MPM vs. HC	AEx vs. HC	MPM	AEx	HC	
P1	27.4	0.576	82 ↓	80 ↓	-	-	-0.0420 (-0.0526; -0.0232)	-0.0222 (-0.0315; -0.0170)	-0.0250 (-0.0450; 0.0006)	*0.042*
P3	6.3	0.586	100 ↑	96 ↑	-	12 ↓	0.1304 (0.0452; 0.2098)	0.0156 (0.0104; 0.0300)	0.0822 (0.0045; 0.2079)	*0.001*
P5	4.9	0.515	100 ↓	100 ↓	-	100 ↑	-0.0570 (-0.1198; -0.0331)	0.0257 (0.0073; 0.0650)	-0.0245 (-0.0636; -0.0069)	*<0.001*
P8	7.6	0.502	20 ↓	9 ↓	-	100 ↑	0.0356 (0.0249; 0.0602)	0.0801 (0.0561; 0.1272)	0.0336 (0.0033; 0.0636)	*<0.001*
P13	21.5	0.717	-	2 ↑	-	100 ↓	0.0002 (-0.0047; 0.0012)	-0.0138 (-0.0212; -0.0041)	-0.0010 (-0.0033; 0.0016)	*<0.001*
P25	9.1	0.516	-	11 ↓	-	100 ↑	0.0014 (-0.0375; 0.0181)	0.0480 (0.0278; 0.0722)	-0.0026 (-0.0227; 0.0030)	*<0.001*
P30	17.2	0.694	21 ↑	98 ↑	-	-	-0.0023 (-0.0043; 0.0001)	-0.0133 (-0.0243; -0.0039)	-0.0011 (-0.0061; 0.0008)	*<0.001*
P33	7.5	0.529	79 ↓	-	-	-	-0.0088 (-0.0125; -0.0005)	0.0011 (-0.0039; 0.0047)	-0.0042 (-0.0096; 0.0012)	*0.023*
P35	68.7	0.598	61 ↓	-	-	-	-0.0024 (-0.0047; 0.0005)	-0.0040 (-0.0075; -0.0015)	-0.0020 (-0.0041; 0.0008)	*0.177*
P45	11.1	0.577	89 ↓	91 ↓	-		-0.0047 (-0.0259; 0.0031)	0.0076 (0.0018; 0.0173)	0.0049 (-0.0106; 0.0178)	*0.006*
P50	78.1	0.730	100 ↑	93 ↑	100 ↑	-	-0.0003 (-0.0014; 0.0011)	-0.0067 (-0.0155; -0.0006)	-0.0070 (-0.0142; -0.0019)	*<0.001*
P54	9.3	0.833	17 ↓	93 ↓	-	-	-0.0010 (-0.0024; -0.0002)	-0.0004 (-0.0014; 0.0008)	0.0006 (-0.0012; 0.0029)	*0.042*
P57	25.6	0.824	67 ↓	-	16 ↓	-	-0.0015 (-0.0031; 0.0000)	-0.0017 (-0.0034; 0.0000)	-0.0008 (-0.0013; 0.0007)	*0.101*
P65	14.5	0.626	-	-	-	53 ↓	-0.0106 (-0.0328; -0.0019)	-0.2267 (-0.4511; -0.0309)	-0.0167 (-0.0382; -0.0043)	*0.001*
P68	7.3	0.632	67 ↓	53 ↓	-	2 ↑	0.0003 (-0.0018; 0.0035)	0.0043 (0.0022; 0.0062)	-0.0003 (-0.0026; 0.0031)	*0.004*
P71	9.5	0.729	97 ↓	96 ↓	16 ↓	-	-0.0070 (-0.0106; -0.0019)	-0.0009 (-0.0057; 0.0039)	0.0035 (-0.0024; 0.0702)	*<0.001*
P84	31.8	0.637	2 ↓	-	100 ↓	-	-0.0051 (-0.0108; -0.0019)	-0.0004 (-0.0041; 0.0045)	0.0010 (-0.0018; 0.0099)	*<0.001*

[a]Median (Q1; Q3). [b]Kruskal-Wallis test.

↓: an increase in signal will result in a lower odds to be classified in the MPM group (first 3 columns) or AEx group (4th column). ↑: an increase in signal will result in a higher odds to be classified in the MPM group (first 3 columns) or AEx group (4th column). $1/K_0$: inverse reduced ion mobility. AEx: Asymptomatic former asbestos workers. HC: Healthy non-asbestos exposed control persons. MPM: Malignant pleural mesothelioma patients. RT: Retention time. V: Volt.

Occupationally asbestos-exposed workers can have a mesothelioma lifetime risk as high as 10% [28]. Therefore, we examined whether breath analysis could be used to discriminate former asbestos-exposed workers from MPM patients and hence, as a potential screening tool. This discrimination had 87% (74%-94%) accuracy, 87% (69%-97%) sensitivity, 86% (67%-96%) specificity, 87% (69%-97%) PPV and 86% (67%-96%) NPV with an AUC_{ROC} of 0.86 (0.74-0.94) (Table 3, Figure 2(b)). The VOCs P3, P5, P30, P50, P54 and P71 were selected most often (Table 4).

With 91% (79%-97%) accuracy, AEx persons could be discriminated from HC controls using the same variables, yielding 95% (80%-100%) sensitivity, 86% (66%-96%) specificity, 88% (70%-98%) PPV and 95% (77%-100%) NPV. The AUC_{ROC} was 0.94 (0.84-1.00) (Table 3, Figure 2(b)). The VOCs P5, P8, P13 and P25 were found to be important in discriminating HC from AEx individuals (Table 4).

HC persons were discriminated from MPM patients with an accuracy of 82% (68%-91%) and an AUC_{ROC} of 0.74 (0.55-0.89), 96% (80%-100%) sensitivity, 67% (45%-84%) specificity and the PPV and NPV were respectively 76% (58%-89%) and 93% (71%-100%) (Table 3, Figure 2(b)). Next to P50, the VOC selected as being the most important feature for the discrimination was P84 (Table 4), which was found to be important only in this discrimination.

DISCUSSION

Using breath analysis by MCC/IMS, we improved the accuracy of discriminating MPM patients from AEx and HC persons. We identified VOCs P3, P5, P50 and P71 as the most important discriminating features between MPM patients and both the control groups, with 87% sensitivity, 70% specificity and a respective PPV and NPV of 61% and 91%.

Considering that asbestos-exposed individuals have an increased risk of developing MPM, early-stage MPM detection is important to explore new perspectives for treatment and improve prognosis. We discriminated AEx persons from MPM patients with 87% accuracy and found some of the same VOCs (P3, P5, P50 and P71) to be the most important features for this discrimination, strengthening their importance for MPM detection. We found other VOCs important in discriminating HC from AEx persons (P8, P13 and P25), suggesting a link of these with asbestos-exposure. The VOC P50 was also found to be important for discriminating MPM patients from healthy controls and, together with the VOC P3, from AEx persons, suggesting a link of the VOCs P3 and P50 with mesothelioma development.

Our results are in line with what has been previously described concerning breathomics for detecting asbestos-related diseases [15-17, 29]. The group of de Gennaro compared breath samples of 13 MPM patients, 13 former asbestos workers and 13 healthy non-exposed controls using gas chromatography-mass spectrometry (GC-MS) [15] and distinguished these three groups with 97.4% accuracy. They found cyclohexane to be the only compound that discriminated MPM from the other groups and cyclopentane to be a marker for long-term asbestos exposure. Since we do not know the inverse reduced ion mobility and retention time for both compounds, we cannot exclude them from our selected VOCs and, hence, it is possible these compounds were also selected in our lasso regression models. Furthermore, Dragonieri *et al.* [16] used an electronic nose (e-Nose) to compare breath samples of the same three groups and distinguished MPM patients from AEx individuals with 92.3% sensitivity and 85.7% specificity and from healthy controls with 92.3% sensitivity and 69.2% specificity. Chapman *et al.* repeated this e-Nose study in 20 MPM patients, 42 healthy controls and 18 persons with asbestos-related diseases and distinguished MPM patients with 90% sensitivity and 88% specificity [17].

These studies show that breath analysis is promising for MPM detection. However, although GC-MS is the gold standard which allows VOCs to be identified with a high sensitivity, it is an expensive, time-consuming and offline technique requiring specific operator training and

laboratory conditions and involves different analytical steps. Furthermore, e-Noses are not specifically built for medical diagnostics and recognize the bulk of the breath as a smellprint but do not identify the individual VOCs, despite their ease for analysis.

We used MCC/IMS as an analysing tool which allows an online sampling where the patients breathe directly into the device and is relatively cheap and user-friendly. It contains a built-in library where the inverse reduced ion mobility and retention time characteristics have been allocated to a specific VOC due to GC-MS crosschecking and MCC/IMS analysis of a VOC standard. This library thus allows to identify VOCs, if validated. This database is constantly being updated with new VOC allocations by GC-MS crosschecking and hence, gives the opportunity to detect biochemical pathways that mark MPM pathogenesis [13].

Cakir *et al.* used MCC/IMS to discriminate 25 patients with either asbestosis and/or asbestos-related pleural thickening from 12 healthy controls based upon exhaled breath [29]. They found a higher alpha-pinene concentration in the diseased group than in the control group and discriminated these groups with 96% sensitivity, 50% specificity, 80% PPV and 86% NPV. They linked alpha-pinene to the asbestos-induced chronic inflammation. However, the authors did not include MPM patients. To our knowledge, we are the first to show that MCC/IMS can be used to discriminate MPM patients from controls.

In contrast, alpha-pinene was not identified as being important in our study. This could be explained by several interstudy differences. First, Cakir *et al.* used an MCC/IMS with a 550 MBq radioactive source for ionization whereas we used a 95 MBq source, meaning they have a larger linear range for VOC ionization and are potentially able to ionize more VOCs. Secondly, they did not include MPM patients, females, (ex-)smokers or asymptomatic former asbestos workers. Their patients with asbestos-related diseases are slightly older than our MPM patients (73 vs. 66 years) and our AEx persons (73 vs. 56 years). Also, their healthy controls were more than half the age of their group with asbestos-related diseases (73 vs. 36 years).

Furthermore, they did not correct for possible exogenous contamination. It is known that hospital air is contaminated with ethanol and acetone and that exogenous VOCs from smoking can influence the breath composition [30], suggesting the need to correct for these contaminants. We tried to counteract environmental contamination by calculating the alveolar gradient [23] and used background-corrected values for analysis. Nevertheless, background correction by calculating the alveolar gradient may not be sufficient to completely remove environmental VOCs. Long-term exposure to exogenous VOCs could lead to storage

in muscle or fat tissue depending on the VOCs' concentration, exposure duration, physicochemical properties and the blood:gas and blood:fat partition coefficient [31], indicating that VOCs can take a long time to be eliminated from the body. Since alpha-pinene is also known to be emitted from several aromatic plants and trees [32], to be present in several teas and pine nuts [33] and to be used as an aromatic in different essential oils and healthcare products [34], it could be that patients were exogenously exposed to alpha-pinene by using these products and hence, alpha-pinene is more likely to be a contaminant from an exogenous source rather than endogenously linked to asbestos exposure. Lastly, the fact that we discriminated MPM from AEx and HC or that we have performed lasso regression instead of a rank sum test and decision tree can be incriminated.

Even though our results are promising to use breath analysis for screening, our study has limitations. The participant numbers are too low to extrapolate the results to the whole at-risk population. Therefore, external validation of our results is mandatory in an independent validation set also including patients with lung cancer and benign asbestos-related diseases. Furthermore, the groups were not age-matched and we found differences in smoking status between the groups. This age difference can be explained by the fact that MPM is a disease with a long asymptomatic latency period leading to a diagnosis at advanced age and that it is hard to find healthy controls without comorbidities at matched age. Although some studies suggest that aging has an effect on human metabolism and VOCs [35, 36], and thus may be a confounding variable, several other groups found VOCs not to be influenced by age [37-39]. We decided not to include age as a predictor in our models, because the significant age difference between the groups is a consequence of the patient selection process. Including age as a predictor would likely make age turn up as an important predictor without any guarantee that it is associated with MPM. Consequently, the obtained predictive quality of a model including age would be overly optimistic. The difference in smoking status is also reflected by differences in pack years. Former asbestos workers and MPM patients are individuals who worked as blue-collar labourers at asbestos-processing factories where the incidence of smoking is known to be higher than in other industries [40-42]. As smoking status was never selected in the lasso model, it is likely that smoking is not predictive for mesothelioma. However, the effect of smoking should be further investigated because smoking can induce the CYP450 enzymes to degrade the VOCs and hydroxylate alkanes to

alcohols which can be further metabolized to aldehydes [43]. Next, since our Teflon transfer line was not heated, it is possible some VOCs were retained at the surface of the tubing and lost for analysis. Finally, although MCC/IMS allows identification of VOCs, we were not yet able to identify our VOCs P3, P5, P50 and P71 by cross-checking with the existing MCC/IMS database and the participant numbers are too low to be certain to identify the molecular discriminators or to say something about the kinetics of these compounds. Furthermore, since we do not have GC-MS supporting data, it could be that the compounds we found are co-eluting compounds or that the compounds are monomers, dimers or trimers of the same molecular compound. So our results must be interpreted carefully. To rule out these technical issues, GC-MS supporting data are needed.

Therefore, future research should take breath samples for gas chromatography-mass spectrometry analysis taken in parallel which will allow us to ultimately identify these VOCs and to find links to the underlying MPM pathogenesis.

CONCLUSION

In conclusion, we demonstrated the feasibility of an easy to perform breath test to discriminate MPM patients from patients with and without occupational asbestos exposure using MCC/IMS. This allows an easy, non-invasive, large-scale enrichment of former asbestos workers at risk for developing MPM for follow-up with repeated imaging. Hence, the acceptable specificity and NPV of our results could hold promise to use this breath test for screening of asbestos-exposed asymptomatic seniors. Results should be further validated in a larger patient population and be compared to other lung diseases before clinical implementation.

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Supplementary Figure 1:

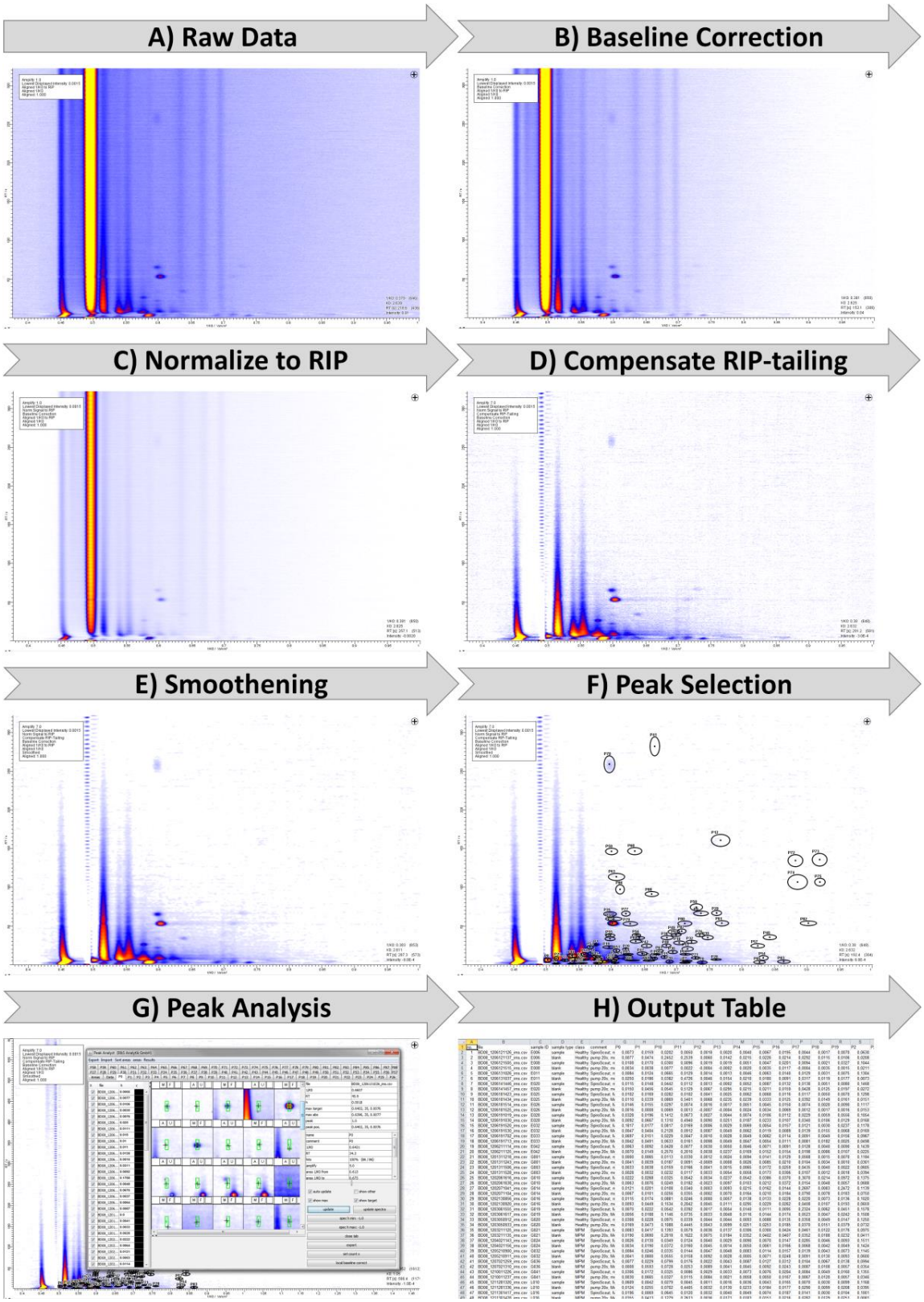

Figure S1: Detailed data analysis scheme. **A:** Raw data. **B:** Data after baseline correction. **C:** Data after normalizing to the reactant ion peak (RIP). **D:** Compensating for RIP-tailing. **E:** smoothening of the data. **F:** Manual selection of 89 volatile compounds in breath and background samples. **G:** Optimization of peak selection and analysis of the maximum peak intensity. **H:** Output file with the maximum intensities per VOC per sample.

CHAPTER 2

Ion mobility spectrometry for screening for malignant pleural mesothelioma: a validation study.

Lamote K, Vynck M, Thas O, Van Cleemput J, Nackaerts K, van Meerbeeck JP. *European Respiratory Journal.* 2017. *Submitted.*

ABSTRACT

Background: Malignant Pleural Mesothelioma (MPM) is predominantly caused by previous asbestos exposure. It is often diagnosed in advanced stages restricting any therapeutic options. Breath analysis can be explored as early detection tool since breath contains volatile organic compounds (VOCs). Using multi-capillary column-ion mobility spectrometry (MCC/IMS) as analysing tool, we previously were able to discriminate MPM patients from asymptomatic persons previously exposed to asbestos fibres and aim to validate our findings and determine the specificity of the model for MPM by comparing with the breath of lung cancer patients.

Methods: Breath and background samples of 52 MPM patients, 59 asymptomatic former asbestos (AEx) workers, 52 healthy non-asbestos exposed persons, 41 patients with benign asbestos-related diseases (ARD), 70 patients with benign non-asbestos-related lung diseases (BLD) and 56 lung cancer (LC) patients were taken for analysis. After background correction, we performed a logistic lasso regression to select the most important VOCs, followed by ROC analysis.

Results: MPM patients could be discriminated from HC, AEx, ARD, BLD and LC patients with 65%, 88%, 82%, 80% and 72%, respectively. When AEx and ARD patients were combined, the discriminating accuracy was 85%. The VOCs selected as most important were P1, P3, P7, P9, P21, P15 and P26.

Conclusion: We discriminated pleural mesothelioma patients from at risk subjects with great accuracy. The high sensitivity and negative predictive value allows to use breath analysis as screening tool for MPM.

Belgian registration number B670201111954

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a tumour from the serosal linings of the thorax, predominantly caused by previous asbestos exposure [1, 2]. Despite the ban on the use of asbestos in Europe in 2005, asbestos is still being mined and consumed by countries in need for industrial growth, remaining an important health issue. Together with the large amount consumed in the past and the long latency period between first exposure and diagnosis of 40-50 years, its incidence is expected to further increase in the future. MPM is usually diagnosed in an advanced stage from a tissue sample often to be obtained through an invasive and costly biopsy, limiting curative treatment options [3]. This results in low survival rates with a median overall survival of up to 18 months with standard of care platinum-based chemotherapy in combination with bevacizumab [4, 5]. Together, this stresses the urgent need for screening tools for early detection, which is believed to improve the patients' outcome.

Breathomics can be used to analyse volatile organic compounds (VOCs) in breath which can serve as markers for MPM [6]. Proof-of-principle studies have identified cyclohexane to discriminate breath samples of 13 MPM patients, 13 occupational asbestos-exposed persons and 13 healthy non-exposed controls using gas chromatography-mass spectrometry (GC-MS) with 97.4% accuracy [7] or used pattern recognition with cross-reactive sensor technology (electronic nose) [8, 9] to discriminate MPM patients from asymptomatic persons with previous occupational asbestos-exposure (AEx) with acceptable accuracy. Using ion mobility spectrometry (IMS), Cakir *et al*. separated patients with benign asbestos-related diseases (ARD) from healthy controls with 99.9% accuracy [10]. Using the same technique, our research group was able to discriminate 23 MPM patients from 21 healthy controls (HC) and 22 AEx patients with 82% and 87% accuracy, respectively [11]. The goal of this study was to validate our findings in a larger study and determine how specific breath analysis is for MPM compared to lung cancer.

MATERIALS AND METHODS

Study design and participants

This study is a multicentre, cross-sectional, case-control study. The study was approved by the Institutional Review Board of Ghent University Hospital (LONG 11-01; Belgian registration number B670201111954) and was conducted in accordance with the Helsinki Convention. Before inclusion, participants had to give their written informed consent. Healthy controls (HC), patients with benign asbestos-related diseases (ARD), patients with benign lung diseases (BLD) unrelated to asbestos exposure, primary lung cancer (LC) patients and pleural mesothelioma (MPM) patients were recruited via the University Hospitals of Ghent, Leuven and Antwerp (Belgium). Other ARD patients and asymptomatic persons formerly exposed to asbestos fibres (AEx) were recruited via the occupational health service of a company that produced asbestos until 1997. MPM diagnosis must be confirmed by the Belgian Mesothelioma Pathology Panel. If any anti-tumour treatment was started before the breath sampling in MPM or lung cancer patients, patients were excluded. Furthermore, other asbestos-related diseases must not be present in any of the control groups except for the ARD patients. A recent CT-scan or chest X-ray (<12 months) was mandatory to confirm the medical condition. Participants were asked to refrain from eating, drinking and smoking at least for 2 hours before the breath sampling. Participants had to fill in 2 questionnaires: one to check if the inclusion criteria were met and one to collect demographical data and previous asbestos exposure data. For patients, a detailed patient record had to be available with all details about the patient's medical condition.

Breath Sampling and Analysis

As breath analysis device, a BioScout multicapillary column-ion mobility spectrometer (MCC-IMS) was used (B&S Analytik, Dortmund, Germany). For breath sampling, a SpiroScout ultrasound-controlled breath sampler (Ganshorn Medizin Electronic, Niederlauer, Germany) was connected to the sample loop of the MCC-IMS. This detects the CO_2-levels in breath and takes a sample when a plateau in CO_2-levels is reached, sampling breath from the alveolar region.

The MCC-IMS characteristics and characteristics are previously described [6, 11]. In short, breath samples were taken between January, 1st 2012 and December, 20th 2014. All participants were asked to first rinse their mouth with distilled water and put on rubber gloves

and a nose clip. Next, while sitting in an upright position at rest and without performing forced breathing manoeuvres, they were asked to breathe normal for 3 minutes through the SpiroScout's mouthpiece, connected to a bacteria filter and the MCC/IMS sample loop. After 3 minutes, 10 ml of alveolar air is sampled in the sample loop using an internal pump and subsequently sent to the MCC-IMS for analysis. The breath analytes first pass a non-polar OV-5 MCC column (Multichrom Ltd, Novosibirsk, Russia) for pre-separation based upon the analytes' chemical characteristics. After passing the MCC column, the pre-separated volatiles enter the ionization chamber of the IMS with a certain retention time. For ionisation of the VOCs, the MCC-IMS uses a 95MBq ^{63}Ni β-radiation source. This ionises a carrier gas (α_1-nitrogen gas, Air Liquide Medical, 99.999% pure, CAS-n°: 7727-37-9, Schelle, Belgium) which will ionise and positively charge the VOCs through secondary ionization and charge transfer reactions. Subsequently, the ionised breath compounds enter a drift tube where a second separation takes place based upon their ion mobility characteristics (size, charge, mass and shape) under the influence of an electrical field and the same α_1-nitrogen gas as counter gas. Finally, the VOCs collide on a Faraday plate detector, evoking an electrical current, which results in a VOC peak intensity (Volt, V) that correlates to its concentration. The drift time from entering the drift tube until collision at the Faraday detector is also measured. After taking a breath sample, a background sample was taken using the same materials and conditions. In order to rule out external contamination or sampling artefacts, we used disposable mouthpieces and filters. All the sample lines are made of Teflon (PTFE), an inert material known not to retain compounds [12]. Between the breath sampling of different participants, the MCC/IMS was flushed at least 10 times with humid air to remove contaminants and to make sure that MCC/IMS spectra were clean.

Statistics

VOC analysis was done as with VisualNow v3.7 software (B&S Analytik, Dortmund, Germany) as previously described [11]. The raw data consists of IMS-chromatograms which separates VOCs by retention time from the MCC column (s) and their inverse reduced ion mobility linked to the drift time in the IMS (Vs/cm²). Next, the chromatograms were pre-processed by aligning all chromatograms and denoising the data through baseline correction using a 5x3 low pass filter [13]. The data is subsequently normalized to the reactant ion peak (RIP), which is the output from the ionised carrier gas, by estimating its shape and subtracting it from the

measured spectra. We compensated for RIP-tailing by subtracting a median spectrum form each spectrum within the data set [13]. Next, the data is smoothed. After a visual inspection of all breath and background samples, 250 VOCs were manually selected and subsequently analysed resulting in a list of VOC-peak intensities (maximum peak height in the selected peak area).

To remove an effect of environmental chemical confounders, the alveolar gradient was calculated for every VOC by subtracting the standardized peak intensity in the background samples from the standardized peak intensity in the corresponding breath samples [14]. These alveolar gradient intensities (Volt, V) are then used in R (version 3.3.1) [15] as predictors together with the patient characteristics and clinical data. Because of the high dimensionality setting (large number of variables and low number of samples), penalized logistic regression (lasso) was used to discriminate MPM patients from asymptomatic former asbestos workers (AEx) and healthy controls (HC) as described earlier [11]. We used the *glmnet* R-package (version 2.0-2) for fitting a binomial lasso logistic model [16]. Using the predicted outcomes of all of the patients, we then constructed a ROC curve and estimated sensitivity, specificity, positive (PPV) and negative predictive value (NPV) and the diagnostic accuracy of the final model and their 95% confidence intervals. We furthermore had a look at the number of times (the number of folds) a VOC was selected by the lasso regressions. Variables selected in a large proportion of folds (>50%) were considered important. Furthermore, we compared MPM patients to HC, AEx, ARD, BLD and LC controls.

Summary statistics of the continuous variables were calculated. A Fisher's exact test was used to test whether the categorical outcomes were equally likely. For continuous variables, a Kolmogorov-Smirnov test was performed to assess normality and subsequently an ANOVA was performed to assess differences of means in case there was no evidence for deviation from normality. For continuous variables showing deviation from normality, differences in their distribution were assessed using a Kruskal-Wallis test. A p-value below 5% was considered statistically significant after adjustment using the Bonferroni procedure.

RESULTS

Patient characteristics

In total, 330 participants were included in the study: 52 healthy controls (HC), 59 asymptomatic persons with a past occupational asbestos-exposure (AEx), 41 patients with benign asbestos-related diseases (ARD), 70 patients with benign lung diseases independent of asbestos-exposure (BLD), 56 primary lung cancer (LC) patients and 52 mesothelioma (MPM) patients (Table 1). There were significantly more males in the groups related to asbestos-related diseases (MPM, AEx, ARD) compared to the other groups and the patient groups were significantly older than the HC and AEx group. There were more current smokers in the AEx, ARD, BLD and LC groups.

Table 1: Baseline patient characteristics.

	HC	AEx	ARD	BLD	LC	MPM	p-value
N	52	59	41	70	56	52	
Gender							
Male	34 (65%)	58 (98%)	40 (98%)	47 (67%)	37 (66%)	43 (83%)	<0.001
Female	18 (35%)	1 (2%)	1 (2%)	23 (33%)	19 (34%)	9 (17%)	
Age (years)							
Median	51.2	53.2	58.3	58.8	69.9	67.3	<0.001
(Q1-Q3)	(34.5-56.7)	(50.2-55.3)	(55.3-62.2)	(40.6-68.0)	(64.3-72.7)	(61.6-72.9)	
Weight (kg)							
Mean	78.3	85.6	84.5	71.3	70.7	74.5	<0.001
SD	17.1	11.4	14.6	15.1	14.4	10.3	
Length (m)							
Mean	1.76	1.77	1.74	1.71	1.68	1.72	<0.001
SD	0.09	0.06	0.05	0.09	0.08	0.08	
BMI (kg/m²)							
Median	25.2	26.9	26.8	24.4	24.0	25.4	<0.001
(Q1-Q3)	(22.2-27.7)	(24.9-28.9)	(24.5-31.5)	(20.8-25.9)	(21.6-27.8)	(23.6-27.2)	
Smoke status							
Never	35 (67%)	19 (32%)	15 (37%)	24 (35%)	6 (10%)	19 (37%)	
Current	1 (2%)	14 (24%)	8 (20%)	14 (20%)	25 (45%)	5 (9%)	<0.001
Ex	16 (31%)	26 (44%)	18 (43%)	31 (45%)	25 (45%)	28 (54%)	
Pack years							
Median	0.0	6.0	5.3	7.5	30.0	2.65	<0.001
(Q1-Q3)	(0.0-1.61)	(0.0-21.5)	(0.0-24.8)	(0.0-36.0)	(14.4-45.0)	(0.0-14.7)	

AEx: asymptomatic former asbestos-exposed individual. ARD: patients with benign asbestos-related diseases. BLD: patients with benign non-asbestos related lung diseases. HC: Healthy control. LC: primary lung cancer patients. MPM: malignant pleural mesothelioma patients.

Breath analysis

MPM patients could be discriminated from HC controls with 65% accuracy (Table 2, Figure 1). Since asbestos is causally linked to MPM pathogenesis and, hence, persons exposed to these fibres are at risk for MPM, we examined if MPM patients could be discriminated from AEx and ARD participants and use it as a screening tool. We discriminated MPM from AEx patients with 88% accuracy, 87% sensitivity, 90% specificity and a PPV and NPV of 88% and 88%, respectively. The AUC_{ROC} was 0.879. MPM patients could be discriminated from ARD patients with 82% accuracy. The sensitivity, specificity, PPV and NPV were 89%, 73%, 81% and 83%, respectively with an AUC_{ROC} of 0.850. Furthermore, pooling both groups allowed to discriminate MPM patients with 85% accuracy, 94% sensitivity, 80% specificity, 71% PPV and 96% NPV. The AUC_{ROC} was 0.890 (Table 2, Figure 1). The large sensitivity and NPV make it an excellent clinical tool for screening and ruling out disease. MPM patients were also nicely discriminated from patients with benign lung diseases with 80% accuracy. The sensitivity, specificity, PPV, NPV and AUC_{ROC} were 71%, 87%, 80%, 80% and 0.837, respectively. However, the discrimination between MPM and LC patients was less clear, showing 72% accuracy, 73% sensitivity, 71% specificity, 70% PPV and 74% NPV. The AUC_{ROC} was 0.770 (Table 2, Figure 1).

Furthermore, with an AUC_{ROC} of 0.522 and accuracy of 55%, we were not able to discriminate AEx from ARD controls, even when a lot of VOCs were included in the models (Table 3, Figure 2). Nevertheless, BLD patients could be nicely discriminated from AEx en ARD patients with 90% and 85% accuracy, respectively.

Lung cancer patients were discriminated from HC controls with 71% accuracy, 87% sensitivity, 65% specificity, 71% PPV and 72% NPV, respectively. Furthermore, AEx participants were also nicely discriminated from LC patients with 90% accuracy. The sensitivity, specificity, PPV and NPV were 89%, 90% 89% and 90%, respectively (Table 3, Figure 2). LC patients were discriminated from BLD patients showing 71% accuracy, 64% sensitivity and 77% specificity. The AUC_{ROC} was 0.724.

By the lasso regression, the most important VOCs selected to discriminate MPM from the at risk groups and BLD patients are P1, P7, P9, P15, P21, and P26. These were not selected when discriminating MPM from HC controls (Table 2). These were also selected to discriminate MPM from LC patients, but this did not generate a large accuracy. Peaks P1, P3, P21 and P26 were also found important to discriminate LC from AEx participants and AEx from BLD patients.

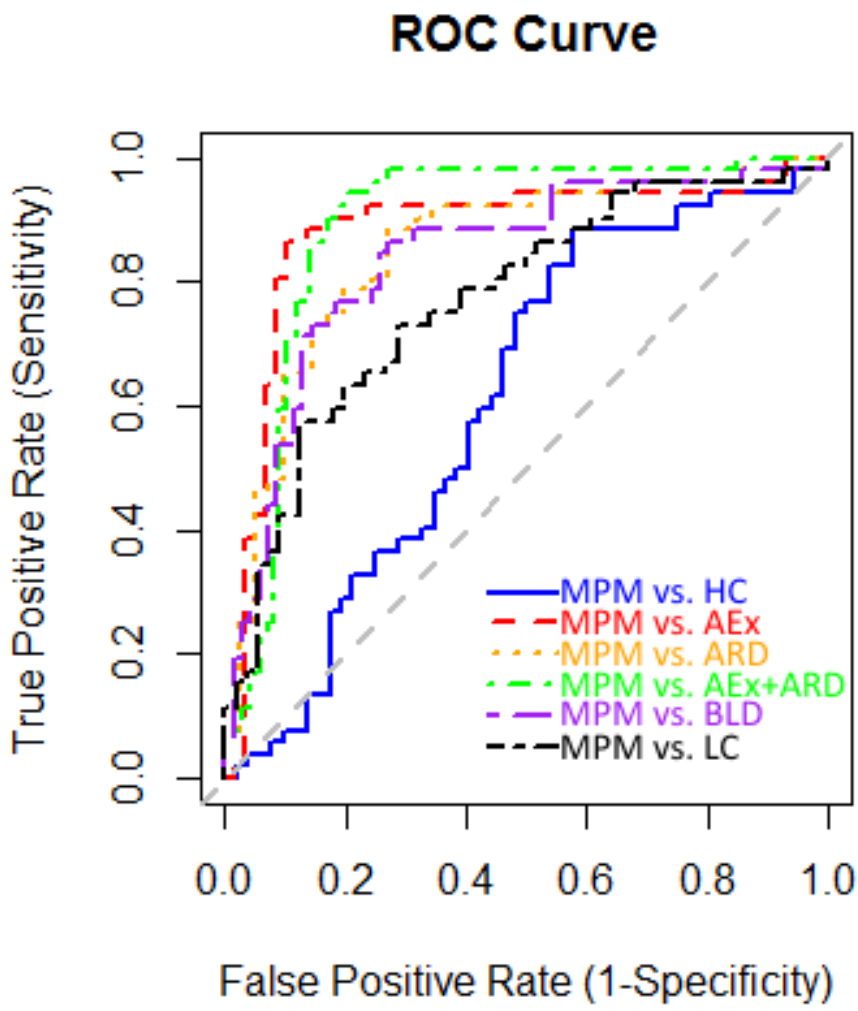

Figure 1: ROC Curves for MPM discrimination. AEx: asymptomatic persons with past asbestos exposure. ARD: patients with benign asbestos-related diseases. BLD: patients with benign non-asbestos-related lung diseases. HC: healthy controls without occupational asbestos exposure. LC: lung cancer patients. MPM: malignant pleural mesothelioma patients.

Table 2: Model characteristics for discriminating mesothelioma.

	MPM vs. HC	**MPM vs AEx**	**MPM vs ARD**	**MPM vs AEx+ARD**	**MPM vs BLD**	**MPM vs LC**
N	*54 vs 52*	*54 vs 59*	*54 vs 41*	*54 vs 100*	*54 vs 70*	*54 vs 54*
Sensitivity	88.5% (77.6%-95.2%)	86.5% (75.2%-93.9%)	88.5% (77.6%-95.2%)	94.2% (85.1%-98.5%)	71.2% (57.8%-82.2%)	73.1% (59.9%-83.8%)
Specificity	42.3% (29.5%-56.0%)	89.8% (80.1%-95.8%)	73.2% (58.2%-85.0%)	80.0% (71.3%-87.0%)	87.1% (77.8%-93.5%)	71.4% (58.7%-82.1%)
PPV	60.5% (49.3%-71.0%)	88.2% (77.2%-95.1%)	80.7% (69.0%-89.4%)	71.0% (59.6%-80.8%)	80.4% (67.2%-90.0%)	70.4% (57.3%-81.4%)
NPV	78.7% (60.7%-90.8%)	88.3% (78.3%-94.7%)	83.3% (68.6%-92.9%)	96.4% (90.5%-99.1%)	80.3% (70.2%-88.1%)	74.1% (61.2%-84.4%)
Accuracy	65.4% (55.9%-74.0%)	88.3% (81.3%-93.3%)	81.7% (72.9%-88.6%)	84.9% (78.5%-89.9%)	80.3% (72.6%-86.7%)	72.2% (63.3%-80.0%)
AUC_{ROC}	0.612 (0.502-0.724)[#]	0.879 (0.799-0.948)[#]	0.850 (0.764-0.927)[#]	0.890 (0.832-0.942)[#]	0.837 (0.759-0.907)[#]	0.770 (0.678-0.855)[#]
VOCs (>50% of times selected)	**P0**, **P4**, **P10**, **P15**, P66, P85, P88, P92, **P99**, **P103**, **P104**, **P108**, **P114**, **P119**, P170, P189, P192, P196, **P203**, **P207**, **P208**, **P212**, **P218**, **P223**	**P1**, **P3**, P7, **P9**, **P15**, **P21**, P22, **P26**, P65, **P66**, P73, P75, **P84**, P99, **P101**, **P110**, **P112**, **P114**, P118, **P120**, **P126**, **P132**, P133, **P137**, P176, **P177**, P184, P186, **P195**, P210, **P212**, **P221**, **P223**, **P225**, P229, **P231**, **P237**, **P243**, P244, **P248**	**P1**, **P9**, P15, **P21**, **P26**, **P34**, **P83**, P88, **P92**, **P94**, **P102**, P108, **P114**, **P119**, **P127**, **P176**, P181, **P185**, **P187**, **P195**, P201, **P207**, **P212**, **P220**	**P1**, **P7**, **P9**, **P15**, **P21**, **P26**, P70, P83, **P84**, **P88**, **P101**, P110, P118, **P122**, P123, P142, **P151**, **P153**, **P159**, **P161**, **P167**, **P173**, **P178**, P222, P235, **P236**, **P240**	**P1**, **P8**, P9, P15, **P42**, P98, P115, **P121**, **P123**, **P130**, P131, P137, **P164**, P220, P237, **P243**, **P245**	**P0**, **P7**, **P8**, **P9**, P15, **P21**, **P28**, **P37**, **P42**, **P43**, **P48**, P64, **P73**, **P78**, **P107**, **P108**, **P115**, **P116**, **P117**, P123, **P129**, **P136**, **P145**, **P150**, **P151**, **P156**, P172, **P181**, **P186**, **P215**, **P216**, **P223**, **P224**, **P225**, P231, **P237**, **P244**

[#]AUC_{ROC} significantly different from 0.50.

AEx: asymptomatic former asbestos-exposed controls. ARD: patients with benign asbestos related diseases. AUC_{ROC}: area under the receiver operator characteristic curve. HC: healthy controls. MPM: malignant pleural mesothelioma. NPV: negative predictive value. PPV: positive predictive value. VOC: volatile organic compound. VOCs in bold are selected in >80% of folds.

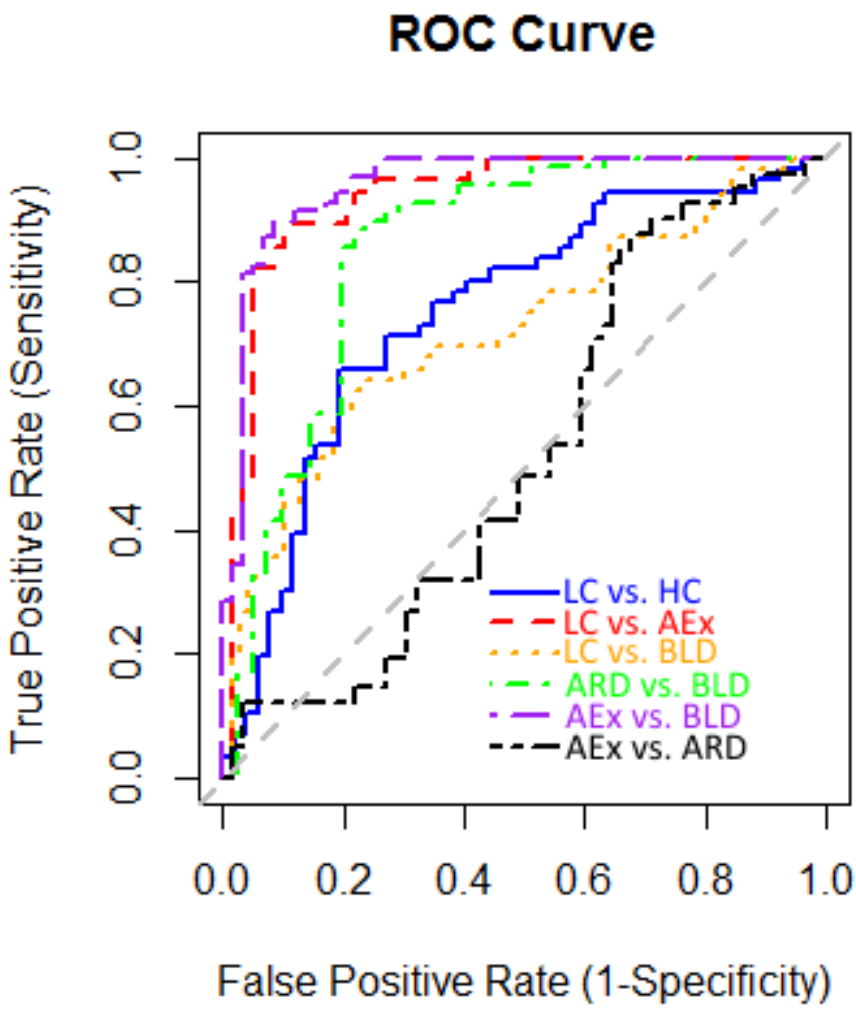

Figure 2: ROC Curves for lung cancer discrimination. AEx: asymptomatic persons with past asbestos exposure. ARD: patients with benign asbestos-related diseases. BLD: patients with benign non-asbestos-related lung diseases. HC: healthy controls without occupational asbestos exposure. LC: lung cancer patients. MPM: malignant pleural mesothelioma patients.

Table 3: Model characteristics for discriminating lung cancer.

	LC vs. HC	LC vs AEx	LC vs BLD	AEx vs ARD	AEx vs BLD	ARD vs BLD
N	*54 vs 52*	*54 vs 59*	*54 vs 70*	*59 vs 41*	*59 vs 70*	*41 vs 70*
Sensitivity	76.8% (64.5%-86.4%)	89.3% (79.1%-95.5%)	64.3% (51.2%-76.0%)	82.9% (69.2%-92.2%)	88.6% (79.5%-94.5%)	88.6% (79.5%-94.5%)
Specificity	65.4% (51.8%-77.3%)	89.8% (80.1%-95.8%)	77.1% (66.3%-85.8%)	35.6% (24.2%-48.4%)	91.5% (82.3%-96.8%)	78.0% (63.6%-88.7%)
PPV	70.5% (58.2%-80.9%)	89.3% (79.1%-95.5%)	69.2% (55.8%-80.6%)	47.2% (36.0%-58.7%)	92.5% (84.3%-97.2%)	87.3% (78.1%-93.6%)
NPV	72.3% (58.4%-83.7%)	89.8% (80.1%-95.8%)	73.0% (62.1%-82.1%)	75.0% (56.7%-88.3%)	87.1% (77.0%-93.8%)	80.0% (65.6%-90.2%)
Accuracy	71.3% (62.3%-79.2%)	89.6% (83.0%-94.2%)	71.4% (63.1%-78.8%)	55.0% (45.2%-64.5%)	89.9% (83.8%-94.3%)	84.7% (77.1%-90.5%)
AUC_{ROC}	0.752 (0.659-0.839)[#]	0.936 (0.884-0.976)[#]	0.724 (0.630-0.813)[#]	0.522 (0.366-0.591)	0.957 (0.917-0.988)[#]	0.855 (0.766-0.930)[#]
VOCs (>50% of times selected)	**P4**, P7, **P8**, **P10**, P23, **P28**, P43, **P55**, P59, **P76**, **P83**, **P107**, **P112**, P115, **P116**, **P118**, P131, **P136**, P151, **P163**, P167, **P184**, **P191**, **P215**, **P220**, P223, **P224**, **P226**, P239, **P244**	P0, **P1**, **P3**, P14, **P21**, **P26**, P43, **P61**, **P65**, **P66**, P72, P84, **P88**, P90, **P101**, P112, **P114**, P115, **P116**, **P118**, P129, **P136**, **P141**, P158, **P176**, **P180**, **P181**, P187, P199, **P203**, P205, **P216**, P227, **P229**, P230, **P231**, **P233**, P244	**P0**, P1, **P42**, P44, P107, **P125**, **P126**, P127, **P168**, P170, P233	P1, **P3**, P20, P23, P26, P34, P37, P44, P66, P69, P70, P80, P83, P84, **P90**, P92, **P99**, P101, **P103**, P120, P123, P126, P134, P137, P144, P166, P169, P170, P180, P183, P184, **P190**, P192, **P199**, P201, **P203**, P223, P226, P234, P237, P244	**P1**, **P3**, **P21**, **P42**, P50, **P84**, **P87**, **P88**, P97, **P101**, P104, **P128**, **P130**, **P132**, **P150**, **P171**, P179, **P213**, **P216**, **P217**, P226, **P230**, P233	**P21**, P25, **P42**, **P87**, **P88**, **P101**, **P110**, **P132**, P136, **P153**, **P198**, **P199**, **P212**, **P221**, **P243**, P247

[#]AUC_{ROC} significantly different from 0.50.

AEx: asymptomatic former asbestos-exposed controls. ARD: patients with benign asbestos related diseases. AUC_{ROC}: area under the receiver operator characteristic curve. HC: healthy controls. MPM: malignant pleural mesothelioma. NPV: negative predictive value. PPV: positive predictive value. VOC: volatile organic compound. VOCs in bold are selected in >80% of folds.

DISCUSSION

In this multicentre, cross-sectional, case-control study, we showed that breath analysis by MCC-IMS discriminated MPM patients with clinical importance from healthy controls, AEx persons, ARD patients, BLD patients, and LC patients with clinically important accuracy. We are the first to perform such a study including a large number of patients and control groups. Persons previously exposed to asbestos fibres have a lifetime increased risk of developing MPM. Together with the poor prognosis, it is of utmost importance to detect the disease in its early stages by screening, which is believed to improve the patient's outcome. Therefore, we examined if MPM patients could be discriminated from asymptomatic persons with past occupational asbestos-exposure and from patients with benign asbestos-related diseases. MPM patients were discriminated from AEx and ARD patients with 88% and 82% accuracy, respectively. When both groups were combined, the accuracy was 85%, with a sensitivity, specificity, PPV and NPV of 94%, 80%, 71% and 96%, respectively. The high sensitivity and NPV from discriminating MPM from these at risk control groups stresses the screening capability. The most important VOCs found in these discriminations are the VOCs P1, P3, P7, P9, P15, P21, and P26 of which P3 was also found in our previous proof-of-principle study [11].

Our results confirm and extend previous studies. The group of de Gennaro *et al.* discriminated 13 MPM patients from 13 AEx and 13 HC persons with 97.4% accuracy [7]. They identified cyclopentane as marker for long-term asbestos-exposure and cyclohexane as marker for MPM. Using pattern recognition, Dragonieri *et al.* distinguished 13 MPM patients from 13 AEx and 13 HC controls with 80.8% and 84.6% accuracy, respectively [8]. The group of Chapman *et al.* repeated this last study and discriminated 10 MPM patients from 32 HC subjects and 18 ARD patients with 88% accuracy, respectively [9]. Using MCC-IMS, Cakir *et al.* discriminated 25 ARD patients from 12 HC with 96% sensitivity and 50% specificity and identified alpha-pinene and 4-ethyltoluol as markers for asbestos-related diseases [10].

Our group used MCC-IMS and discriminated 23 MPM patients from 22 AEx and 21 HC subjects with 87% and 82% accuracy, respectively. We found P3, P5, P50 and P71 as most important VOCs in the discriminations [11].

However, with 72% accuracy, we were not able to fully discriminate MPM patients from LC patients. This could be due to the fact that VOCs are induced by inflammation. Since inflammation is one of the hallmarks in cancer [17], these VOCs could be more general

markers for cancer rather than tumour-specific. However, since we obtained a modest discrimination between MPM and LC and the VOCs used for this discrimination are mostly different from those used to discriminate MPM from the at risk groups, this indicates the possibility that some VOCs are able to discriminate between the different tumour types.

The strength of our study lies within the inclusion and comparison of multiple control groups and the large number of participants included in each group. To our knowledge, we are the first to report the use of breath analysis for MPM including a large number of patients and control groups. Our study serves as the final proof-of-principle and validates all previous research. Despite these satisfying results, we acknowledge our study has some limitations. First, since this is no randomised study, the groups were not matched for gender, age, BMI and smoking status. MPM and LC patients were significantly older and could be explained by the latency period between first exposure to the causal agent and the diagnosis of the diseases. This is further explained by the fact that healthy controls without significant comorbidities or medication use are hard to find at matched age. And although some studies suggest that aging has an effect on human metabolism and VOCs [18, 19], several other groups found VOCs not to be influenced by age [20-22]. Furthermore, a higher incidence of males was seen in the groups with asbestos exposure (AEx, ARD and MPM). This can be explained by the fact that the asbestos-industry is known to have a male predominance. This industry also explains the difference in smoking status: asbestos-workers are operational in the blue-collar industry, which is also known to have a higher incidence of current smokers. Furthermore, since smoking is the main causal agent of lung cancer, it is expected to have the highest incidence of current smokers in our study. However, we do not believe smoking had any impact on the modelling considering MPM pathogenesis is independent of smoking. This is further strengthened by the fact we could not discriminate MPM patients from LC patients. Secondly, although we took background samples for correction, we cannot fully exclude the possibility that external VOCs could have influenced the breath samples. Dependent on their kinetics, VOCs can be inhaled and stored in the body's fat compartments and slowly released over time [23, 24]. Although we have tried to counteract environmental contamination as much as possible by using inert sampling materials and by calculating the alveolar gradients of the VOCs as stated by Phillips *et al.* [14], it is only one way to cope with background effects and may not be sufficient to completely remove the impact of environmental confounders.

However, since the patients were randomly sampled and, hence, also the background samples, the effects of contamination were excluded as much as possible.
Lastly, our selected VOCs have not been identified and are not included in the MCC-IMS VOC library. This has no impact on the discriminating accuracy but identification of the VOCs should allow us to biologically link the VOCs to MPM pathogenesis and serve as additional proof.

In summary, we can say that MPM patients were discriminated from at risk groups with a great clinically relevant accuracy. The large sensitivity and negative predictive value allow breath analysis to be used as step-up screening tool in the diagnostic workflow for MPM. Future research should now focus on the next step in biomarker validation: the external validation in a prospective, case-control, series in independent patient cohorts, with blinding of the investigator for the underlying pathology, and follow at risk subjects over time. This will ultimately lead to assess the clinical utility of the breath test compared to the current imaging screening.

CONCLUSION

Using MCC-IMS, we discriminated pleural mesothelioma patients from healthy controls, subjects previously exposed to asbestos, with benign asbestos-related and non-related lung diseases and lung cancer patients with great accuracy. The high sensitivity and negative predictive value allows to use breath analysis as screening tool for MPM. Validating these results in an independent, blinded prospective study should allow us to assess the clinical utility of breath analysis for MPM screening in persons previously exposed to asbestos fibres.

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CHAPTER 3

Validating breath analysis to screen for pleural mesothelioma: a cross-sectional, case-control study.

Lamote K, Brinkman P, Vandermeersch L, Vynck M, Sterk PJ, Van Langenhove H, Thas O, Van Cleemput J, Nackaerts K, van Meerbeeck JP. *Oncotarget.* 2017. *In revision.*

ABSTRACT

Rationale: Malignant pleural mesothelioma (MPM) is mainly caused by previous exposure to asbestos fibres and has a poor prognosis. Due to a long latency period between exposure and diagnosis, MPM incidence is expected to peak between 2020-2025. Screening of asbestos-exposed individuals is believed to improve early detection and hence, MPM management. Recent developments focus on breath analysis for screening since breath contains volatile organic compounds (VOCs) which reflect the cell's metabolism.

Objectives: The goal of this cross-sectional, case-control study is to identify VOCs in exhaled breath of MPM patients with gas chromatography-mass spectrometry (GC-MS) and to validate these VOCs to screen for MPM using an electronic nose (eNose).

Methods: Breath and background samples were taken from 64 subjects: 16 healthy controls (HC), 19 asymptomatic former asbestos-exposed (AEx) individuals, 15 patients with benign asbestos-related diseases (ARD) and 14 MPM patients. Samples were analysed with both GC-MS and eNose.

Results: Using GC-MS, AEx individuals were discriminated from MPM patients with 97% accuracy, with diethyl ether, limonene, nonanal, methylcyclopentane and cyclohexane as important VOCs. This was validated by eNose analysis. MPM patients were discriminated from AEx+ARD participants by GC-MS and eNose with 94% and 74% accuracy, respectively. The sensitivity, specificity, positive and negative predictive values were 100%, 91%, 82%, 100% for GC-MS and 82%, 55%, 82%, 55% for eNose, respectively.

Conclusion: This study shows accurate discrimination of patients with MPM from asymptomatic asbestos-exposed persons at risk by GC-MS and eNose analysis of exhaled VOCs and provides proof-of-principle of breath analysis for MPM screening.

INTRODUCTION

Malignant pleural mesothelioma (MPM) is an aggressive tumour originating from the pleural lining of the thorax and is causally associated with previous asbestos exposure [1, 2]. Despite a ban on asbestos use in the entire European Union in 2005, asbestos is still being produced and consumed in several countries in need for industrial growth. Together with a long average latency period of 40-50 years between first asbestos exposure and MPM diagnosis, this indicates that MPM incidence will further increase [3]. With a 5-year survival rate below 5%, prognosis remains poor, stressing the need for an earlier diagnosis by screening. Serum biomarkers have not proven to be useful for the screening and diagnosis of MPM [4, 5]. Therefore, recent research focused on breath analysis [6]. Breath contains volatile organic compounds (VOCs) which arise from the body's (patho)physiological processes and have demonstrated to be useful in the detection of asthma, COPD, and tumours [7-12].

Asbestos fibres are known to initiate oxidative stress at the mesothelium [13], inducing lipid peroxidation of the mesothelial cell wall, releasing VOCs, and mutagenic DNA lesions. Furthermore, asbestos fibres activate the NF-κB pathway and promote cell survival which contributes to MPM development [14]. VOCs enter the bloodstream, are transported to the lungs where they enter the alveoli through the gas exchange mechanisms and finally are exhaled. Few studies have addressed the use of VOCs for MPM detection. One study analysed breath samples from 13 MPM patients, 13 occupationally asbestos-exposed persons, and 13 healthy non-exposed controls using gas chromatography-mass spectrometry (GC-MS) [15]. Cyclohexane allowed to discriminate MPM patients with 97.4% accuracy. Two studies used pattern recognition of exhaled VOCs by cross-reactive sensor technology (electronic nose: e-Nose) to compare breath samples of the same 3 groups. Dragonieri *et al.* [16] and Chapman *et al.* [17] distinguished MPM patients from controls with 92.3% and 90% sensitivity, respectively. Recently, our research group was able to discriminate 23 MPM patients from 22 asymptomatic occupationally asbestos-exposed persons and 21 healthy non-exposed controls with 87% sensitivity and 70% specificity using multicapillary column-ion mobility spectrometry (MCC/IMS) [18]. Nevertheless, these studies have not been replicated nor has GC-MS been directly validated against eNose.

Since MPM is linked to asbestos exposure and oxidative stress, we hypothesize that VOCs and VOC patterns will differ between MPM patients, persons occupationally exposed to asbestos, and those unexposed. To that end, we aimed to identify discriminating VOCs by GC-MS and

validate the between-group comparisons with eNose in order to provide the proof-of-principle of screening for MPM by breath analysis.

MATERIALS AND METHODS

Study design and participants

We performed a multicentre, cross-sectional, case-control study in 64 subjects. Fourteen MPM patients, fifteen patients with well-defined benign asbestos-related diseases (ARD), and sixteen healthy non-asbestos exposed (HC) controls were recruited in the three participating university hospitals. Nineteen asymptomatic former asbestos-exposed individuals with well-documented asbestos exposure, were recruited via the occupational health service of a Belgian fibre-cement factory that processed asbestos until 1997. Treatment-naïve MPM patients were included after diagnosis, confirmed by the Belgian Mesothelioma Pathology Panel. Exclusion criteria were the start of any anti-tumour treatment before breath sampling, and the presence of non-asbestos-related diseases in the control groups. Before inclusion, a recent CT scan or chest X-ray (<12 months) had to be present to confirm the medical condition. The study was approved by the Institutional Review Board of Ghent University Hospital (LONG 11-01; Belgian registration number B670201111954) and was conducted in accordance with the Helsinki Convention. Participants had to give their written informed consent and two questionnaires had to be completed: one to check if the participants met the inclusion criteria and one to collect data about demographics and past occupational asbestos exposure. For all patients, a detailed medical record had to be available.

Breath sampling

Breath was sampled using a previously validated method [8, 12]. In short, participants breathed tidally with a nose clip into a 2-way non-rebreathing valve (Hans Rudolph 2700, Hans Rudolph, Kansas City, USA) with an inspiratory VOC-filter (A2, North Safety, Middelburg, NL) at the inlet side. After 5 minutes of tidal breathing, the participants inhaled maximally and the expiratory port was connected to a 10 L Tedlar bag (SKC Inc., Eighty Four, PA, USA). Subsequently, the subjects exhaled a full vital capacity volume into the Tedlar bag which was closed afterwards. Within 10 minutes, the bag was connected to an external pump and 500 ml of the breath sample was loaded onto a sorbent tube (3.5” long, 0.25” outer diameter) filled with 200 mg Tenax®GR (35/60 mesh; Markes International Ltd., Llantrisant, UK) for GC-MS analysis at a flow rate of 100 $ml.min^{-1}$ for 5 minutes. Immediately afterwards, 500 ml of the breath sample was loaded onto another Tenax®GR tube (Tenax®GR SS 6 mm x 7” (CAMSCO,

Houston, Texas, USA)) for eNose analysis at a flow rate of 250 ml.min^{-1} for 2 minutes. The sampling tubes were tightened, packed in a glass jar, and sent out for central analysis.

Gas chromatography – mass spectrometry (GC-MS) analysis

Prior to use, the Tenax®GR-tubes were conditioned for 1 hour at 300°C while being flushed with helium (50 ml.min^{-1}). After conditioning but before sampling, the tubes were loaded with 10.7 ng toluene-d8 internal standard, by making a two-phase system and using a home-made injector system. After sampling, breath analytes were desorbed from the Tenax®GR-column using a Unity series 2 Thermal Desorption system (Markes, Llantrisant, UK) by heating the tube to 260°C (10 min at 20 ml.min^{-1}). Prior to desorption, tubes were dry purged for 4 minutes at 20 ml.min^{-1}. Next, analytes were refocused on a microtrap filled with Tenax®TA, cooled at -10°C. After flash-heating the microtrap at 280°C for 3 min, analytes were carried by a He-flow (constant pressure: 50 kPa) and injected with a split-flow of 5 ml.min^{-1} onto a 30 m FactorFour VF-1ms low-bleed bounded-phase capillary GC-column (Varian, Sint-Katelijne-Waver, Belgium; 100% polydimethylsiloxane, internal diameter 0·25 mm, film thickness 1 mm). The flow path was heated to 130°C. The GC (Focus GC, Thermo Finnigan, Milan, Italy) oven temperature was initially set at 35°C for and kept for 10 minutes, then heated to 60°C at a rate of 2°C.min^{-1}. Afterwards the temperature was increased to 170°C at 8°C.min^{-1} and finally to 240°C (at 15°C.min^{-1}), maintained for 10 min. The MS transfer line was heated to 240°C. The ion source was put at 220°C. Masses with m/z 29 to 300 were recorded in full scan mode (200 ms/scan) on a DSQII Single Quadrupole MS (Thermo Finnigan, Austin, TX, USA), hyphenated to the GC, and operating at an electron impact energy of 70 eV. Chromatograms and mass spectra were processed using XCalibur software (Thermo Finnigan, v2.2) and the NIST database. For unidentified compounds, the Kováts retention index (I_K) was calculated.

Electronic nose (eNose) analysis

Exhaled VOCs were thermally desorbed from Tenax®GR tubes using nitrogen as carrier gas. Next, samples were analysed by an assembly of four different eNoses, based on deviant sensor technologies: Cyranose C320 [19], Tor Vergata eNose [20], Common Invent eNose [21], and Owlstone Lonestar [22]. When exposed to a gas mixture, the sensors swell, resulting in a change of electrical resistances (ΔR). The ΔR/R-values are stored as raw data, producing a breathprint that describes the VOC mixture which can be used for pattern-recognition

algorithms [23, 24]. The final eNose-based breath profiles were established by merging the sensor defections of all four devices.

Statistics

R (v3.3.1) using the R studio interface was used for data analysis. Categorical variables are compared using a Pearson Chi^2-test and reported as ratios. For continuous variables, normality was checked by a Shapiro-Wilk test. Dependent on the outcome, variables are given as mean (standard deviation) or median (quartile 1-quartile 3).

The raw eNose data were reduced by principle components analysis into principle components (PC). PCs explaining at least 70% of variance were retained and subsequently used as independent variables for linear discriminant analysis. The leave-one-out cross-validated (LOOCV) accuracy was reported in order to limit false discoveries. Receiver operating characteristic (ROC) curves were constructed.

For GC-MS data, the high number of variables and the rather low number of samples requires penalized logistic regression using the least absolute shrinkage and selection operator (lasso) to search for VOCs that have the most discriminative power for distinguishing MPM patients from controls. We used the *glmnet* R-package (v2.0-2) for fitting binomial lasso logistic models. This involves the selection of a tuning parameter (λ) that determines the number of selected VOCs. The optimal λ is selected by fitting the model for a sequence of λ-values, and for each of the λ-values the fitted model is evaluated by estimating the misclassification error rate by LOOCV. The λ-value minimizing this error rate was selected and used to fit the final model. Using the predicted outcomes of all of the patients, we then constructed a ROC curve (using the *ROCR* R-package (v1.0-7)) and estimated sensitivity, specificity, positive (PPV) and negative predictive value (NPV), the diagnostic accuracy of the final model, and the area under the curve (AUC_{ROC}) with their 95% confidence intervals. We furthermore examined the number of times a VOC was selected by the lasso regressions. Variables selected in >50% of folds were considered important.

RESULTS

Patient characteristics

Sixty-four participants were included: 14 treatment-naïve MPM patients, 15 patients with benign asbestos-related diseases (ARD), 19 AEx persons, and 16 HC individuals (Table 1). MPM patients were significantly older than the other groups; AEx persons were the youngest. No significant differences were found in smoking status, pack years or BMI between the groups although we observed a trend with AEx persons having have more current smokers. Among the ARD patients, 14 (93%) had pleural plaques and 1 (7%) had asbestosis.

Table 1: Patient characteristics

	HC	AEx	ARD	MPM	*p-value*
N	16	19	15	14	
Gender					
Male	15 (93.8%)	19 (100%)	14 (93.3%)	11 (78.6%)	*0.173*[a]
Female	1 (6.3%)	0 (0.0%)	1 (6.7%)	3 (22.4%)	
Age	56 (52.5 – 59.4)	50 (49.6 – 53.2)	60 (58.3 – 63.8)	69 (65.7 – 73.6)	*<0.001*[b]
Smoke status					
Current	0 (0.0%)	6 (31.6%)	1 (6.7%)	1 (7.1%)	
Ex	8 (50.0%)	7 (36.8%)	5 (33.3%)	9 (64.3%)	*0.079*[a]
Never	8 (50.0%)	6 (31.6%)	9 (60.0%)	4 (28.6%)	
Pack years	0.3 (0.0 - 6.1)	9.0 (0.0 - 36.0)	0 (0.0 – 10.5)	7 (0.0 – 30.0)	*0.106*[b]
BMI (kg/m²)	27 (23.4 – 29.3)	27 (25.4 – 28.4)	27 (24.5 – 32.8)	26 (23.9 – 27.1)	*0.529*[b]

[a]Fisher's Exact test.
[b]non-parametric Kruskal-Wallis test.
AEx: asymptomatic former asbestos-exposed controls. ARD: patients with benign asbestos related diseases. BMI: body mass index. HC: healthy controls. MPM: malignant pleural mesothelioma.

GC-MS analysis

In total, 14 MPM patients, 19 AEx subjects, 15 ARD patients and 14 HC controls gave a breath sample for GC-MS analysis. We analysed 5 different models (Table 2, Figure 1): MPM vs. HC (model 1), MPM vs. AEx (model 2), MPM vs. ARD (model 3), MPM vs. AEx+ARD (model 4) and ARD vs. AEx (model 5). Model 1 showed a diagnostic accuracy of 71% (52.9%-85.7%). The AUC_{ROC} was 0.770.

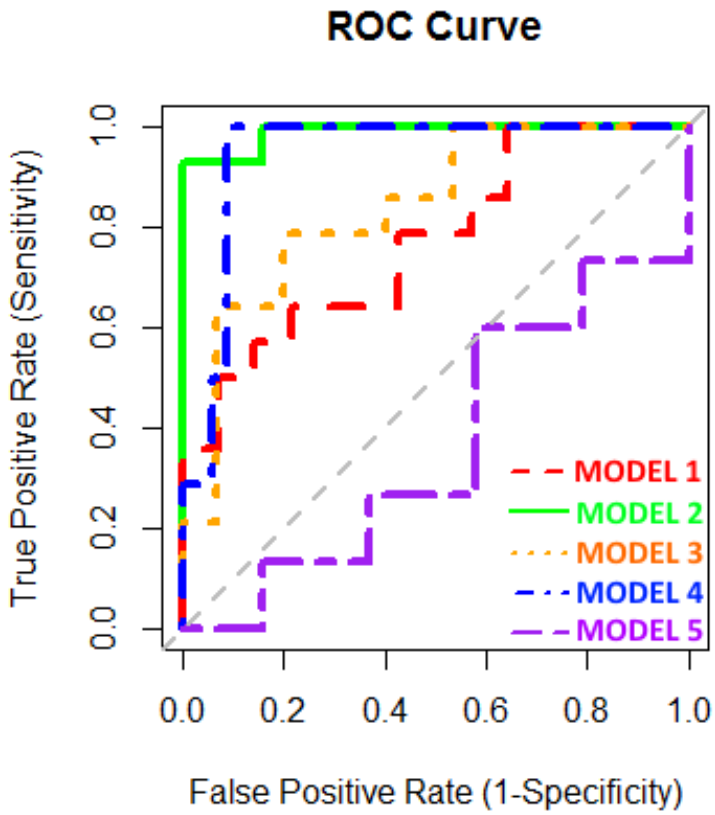

Figure 1: ROC curves of the different models based upon GC-MS analysis.

Since asbestos-exposed individuals can have a lifetime risk of MPM up to 10%,[13] we examined if it was possible to discriminate AEx and ARD participants from MPM patients in view of using it as screening tool (models 2-4). Discriminating MPM from AEx persons was possible with 97% accuracy (86.0%-99.8%), 93% sensitivity, 100% specificity, 100% PPV, and 95% NPV. The AUC_{ROC} was 0.989. Discriminating MPM from ARD patients was possible with 79% accuracy (61.9%-91.2%), 79% sensitivity, 80% specificity, 79% PPV, and 80% NPV. The AUC_{ROC} was 0.838. By pooling ARD and AEx persons, we could discriminate MPM patients with 94% accuracy (84.0%-98.4%), 100% sensitivity, 91% specificity, 82% PPV, and 100% NPV. The AUC_{ROC} was 0.943.

The most frequently selected VOCs in these discriminations were diethyl ether, limonene, cyclohexane, nonanal, VOC I_K 1287 and isothiocyanatocyclohexane (Table 2, Figure S1-S2).

As negative control analysis, we tried to discriminate ARD patients from AEx persons (model 5). This was not possible, showing 50% accuracy (33.6%-66.4%) and an AUC_{ROC} of 0.365, even when important discriminators from the other models were included.

Table 2: Model characteristics from GC-MS data.

	MODEL 1	MODEL 2	MODEL 3	MODEL 4	MODEL 5
Cases vs Controls	***MPM vs HC***	***MPM vs AEx***	***MPM vs ARD***	***MPM vs AEx+ARD***	***AEx vs ARD***
N	***14 vs 14***	***14 vs 19***	***14 vs 15***	***14 vs 34***	***19 vs 15***
Sensitivity	64.3% (37.6%-85.6%)	92.9% (69.5%-99.6%)	78.6% (52.1%-94.2%)	100% (80.7%-100%)	60.0% (34.6%-81.9%)
Specificity	78.6% (52.1%-94.2%)	100% (85.4%-100%)	80.0% (54.7%-94.6%)	91.2% (77.9%-97.7%)	42.1% (21.9%-64.6%)
PPV	75.0% (45.9%-93.2%)	100% (79.4%-100%)	78.6% (52.1%-94.2%)	82.4% (59.2%-95.3%)	45.0% (24.7%-66.7%)
NPV	68.8% (43.7%-87.5%)	95.0% (77.8%-99.7%)	80.0% (54.7%-94.6%)	100% (90.8%-100%)	57.1% (31.2%-80.4%)
Accuracy	71.4% (52.9%-85.7%)	97.0% (86.0%-99.8%)	79.3% (61.9%-91.2%)	93.8% (84.0%-98.4%)	50.0% (33.6%-66.4%)
AUC_{ROC}	0.770 (0.577-0.923)	0.989 (0.955 – 1.000)	0.838 (0.671-0.962)	0.943 (0.866-1.000)	0.365 (0.435-0.818)
VOCs (≥50% of times selected)	Nonane VOC I_K 1349 Propylbenzene Benzonitrile Isoprene Limonene 3-methylpentane 1,3-dichlorobenzene	Ethanol Diethyl ether 2-ethyl-1-hexanol Limonene Nonanal 2-methyl-1-propanol Methylcyclopentane Cyclohexane 1,2,4-trichlorobenzene Naphtalene VOC I_K 679 Phenol Chloroform Linalool Furfural VOC I_K 1287 Bromobenzene	VOC I_K 931 VOC I_K 1493 Beta-pinene Diethyl ether Limonene Hexane 1,2-dichlorobenzene	Ethanol Diethyl ether Isothiocyanatocyclohexane VOC I_K 1233 VOC I_K 1287 VOC I_K 1309 1,2-dichlorobenzene n-Butylbenzene Methylbenzoate 1,2,3-trichlorobenzene Limonene Bromobenzene VOC I_K 1100 Tert-butylbenzene m/p-xylene 2,2,4-trimethylpentane Hexamethyldisiloxane VOC I_K 1493 VOC I_K 720	Limonene Isopropyl acetate 1,3,5-triisopropylbenzene Diethyl ether 3,7-dimethyl-3-octanol Trichloroethylene Dimethyldisulfide Ethanol Phenol Acetophenone 2-methyl-1-propanol 1-butanol Naphtalene VOC I_K 615 1-methylthio-1-propene Isothiocyanatocyclohexane Isopropylbenzene VOC I_K 566 VOC I_K 1111 Hexanal VOC I_K 767

					Ethylbenzene VOC I_K 1349 1,3-dichlorobenzene Dimethylsulfide VOC I_K 1105 2-hexanone VOC I_K 732 Nonane 3-methylpentane n-Butylbenzene

AEx: asymptomatic former asbestos-exposed controls. ARD: patients with benign asbestos related diseases. AUC_{ROC}: area under the receiver operator characteristic curve. HC: healthy controls. I_K: Kováts retention index. MPM: malignant pleural mesothelioma. NPV: negative predictive value. PPV: positive predictive value. VOC: volatile organic compound.

eNose analysis

In total, 11 MPM patients, 15 AEx subjects, 12 ARD patients and 12 HC controls gave a breath sample for eNose analysis. We analysed the same 5 models as with GC-MS analysis (Table 3, Figure 2). We were able to discriminate MPM patients from HC controls (model 1) with 65% accuracy (44.5%-82.3%). The AUC_{ROC} was 0.667. Discriminating MPM from AEx persons was possible with 73% accuracy (53.9%-87.4%), 80% sensitivity, 64% specificity, 75% PPV, and 70% NPV. The AUC_{ROC} was 0.655.

MPM patients could be discriminated from ARD patients with 70% accuracy (48.9%-85.6%), 75% sensitivity, 64% specificity, 69% PPV, and 70% NPV. The AUC_{ROC} was 0.758. When ARD and AEx persons were pooled, we could discriminate MPM patients with 74% accuracy (58.1%-85.8%), 82% sensitivity, 55% specificity, 82% PPV, and 55% NPV. The AUC_{ROC} was 0.747.

Again, it was not possible to discriminate AEx persons from ARD patients, showing 52% accuracy (33.4%-70.0%) and an AUC_{ROC} of 0.550.

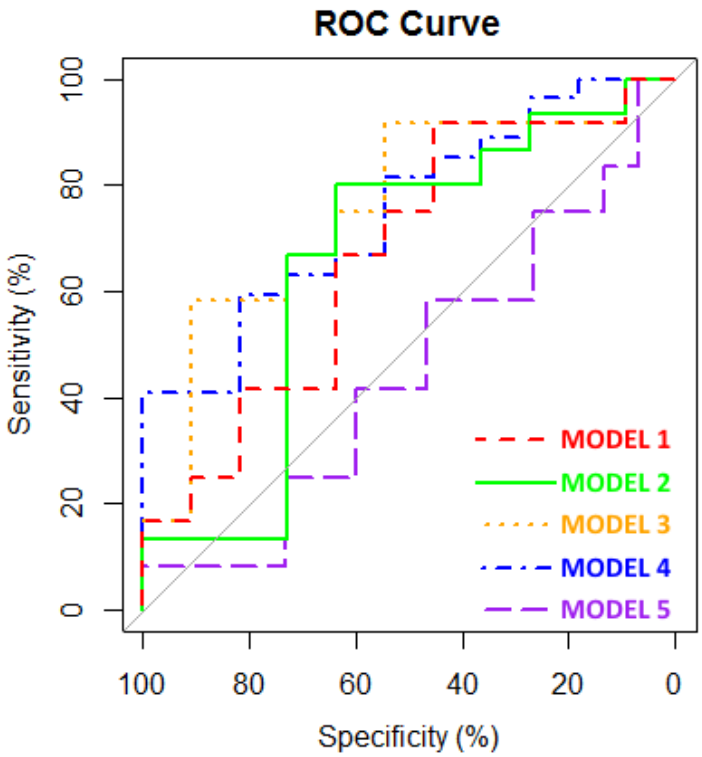

Figure 2: ROC curves of the different models based upon eNose analysis.

Table 3: Model characteristics from eNose data.

	MODEL 1	MODEL 2	MODEL 3	MODEL 4	MODEL 5
Cases vs Controls	***MPM vs HC***	***MPM vs AEx***	***MPM vs ARD***	***MPM vs AEx+ARD***	***AEx vs ARD***
N	***11 vs 12***	***11 vs 15***	***11 vs 12***	***11 vs 27***	***15 vs 12***
Sensitivity	66.7% (37.7%-88.4%)	80.0% (54.7%-94.6%)	75.0% (45.9%-93.2%)	81.5% (63.7%-92.9%)	58.3% (30.3%-82.8%)
Specificity	63.6% (33.7%-87.2%)	63.6% (33.7%-87.2%)	63.6% (33.7%-87.2%)	54.5% (26.0%-81.0%)	46.7% (23.2%-71.3%)
PPV	66.7% (37.7%-88.4%)	75.0% (50.1%-91.5%)	69.2% (41.3%-89.4%)	81.5% (63.7%-92.9%)	46.7% (23.2%-71.3%)
NPV	63.6% (33.7%-87.2%)	70.0% (38.0%-91.7%)	70.0% (38.0%-91.7%)	54.5% (26.0%-81.0%)	58.3% (30.3%-82.8%)
Accuracy	65.2% (44.5%-82.3%)	73.1% (53.9%-87.4%)	69.6% (48.9%-85.6%)	73.7% (58.1%-85.8%)	51.9% (33.4%-70.0%)
AUC_{ROC}	0.667 (0.434-0.900)	0.655 (0.416-0.893)	0.758 (0.548-0.967)	0.747 (0.582-0.913)	0.550 (0.322-0.778)

AEx: asymptomatic former asbestos-exposed controls. ARD: patients with benign asbestos related diseases. AUC_{ROC}: area under the receiver operator characteristic curve. HC: healthy controls. MPM: malignant pleural mesothelioma. NA: not applicable. NPV: negative predictive value. PPV: positive predictive value.

DISCUSSION

In this cross-sectional study, breath analysis by GC-MS allows to discriminate with great accuracy mesothelioma patients from healthy controls, patients with benign asbestos-related diseases, and asymptomatic individuals occupationally exposed to asbestos fibres in the past. This was replicated with an eNose. To our knowledge, this is the first time these discriminations are shown using multiple control groups and taking samples in parallel for GC-MS analysis and eNose replication by using previously validated methods [12]. Considering that occupationally asbestos-exposed persons have a lifetime increased risk for MPM and the latter is a lethal condition with a late-onset development, early detection is of utter importance to improve the disease's management. Therefore, we examined whether it was possible to discriminate MPM patients from AEx and ARD persons. By GC-MS, we discriminated MPM from AEx and ARD persons with 97% and 79% accuracy, respectively. When both groups were pooled, the accuracy was 94%. Given the large sensitivity and NPV of these findings, the present study underlines the capacity of breath analysis as screening tool for persons at risk for MPM.

The most important VOCs selected in all of these GC-MS discriminations are nonanal, diethyl ether, limonene, methylcyclopentane, cyclohexane, a VOC with I_K 1287, and isothiocyanatocyclohexane. Furthermore, AEx persons were not easily discriminated from ARD patients, even when including the VOCs that discriminated MPM from the at risk groups, serving as negative controls. This underlines their importance as breath biomarkers for the presence of MPM.

Our results confirm and extend the findings from previous studies [15-18, 25]. Using GC-MS, de Gennaro *et al.* discriminated 13 MPM patients from 13 HC controls and 13 AEx persons with 97.4% accuracy using cyclopentane, cyclohexane, dodecane, dimethyl nonane, limonene and β-pinene [15]. The group showed cyclohexane to be an important MPM marker and cyclopentane as marker for asbestos-exposure. We also found cyclohexane and limonene to be important in the discrimination of MPM from AEx patients and β-pinene to discriminate ARD from MPM patients. Furthermore, we found diethyl ether and nonanal important discriminators of MPM from AEx and/or ARD patients. These compounds are also found discriminative for lung cancer and are likely to be associated with tumorigenesis [26-28]. This adds to the plausibility of the discriminating capacity of these compounds of MPM.

Furthermore, Cakir *et al.* discriminated ARD patients and HC controls using ion mobility spectrometry with 99.9% accuracy, 96% sensitivity, and 50% specificity based upon α-pinene and 4-ethyltoluol [25]. We did not find these compounds as important discriminators in our models, weakening their importance as markers for ARD.

The GC-MS findings were replicated by pattern recognition of VOCs obtained by the cross-reactive sensors from the eNose. We discriminated MPM patients from HC controls, AEx persons and ARD patients with 65%, 73%, and 70% accuracy, 67%, 80%, and 75% sensitivity and 64%, 64%, and 64% specificity, respectively. When AEx and ARD patients were pooled, MPM patients were discriminated with 74% accuracy, 82% sensitivity and 55% specificity. The finding that the discriminative capacity by the present eNose were somewhat lower as compared to the GC-MS results is due to the smaller number of patients who gave a sample for eNose analysis.

Although we are reaching the same conclusions, our results slightly differ from those previously reported. Using the Cyranose, Dragonieri *et al.* discriminated 13 MPM patients from 13 AEx controls with 92% sensitivity and 86% specificity and from 13 HC controls with 92% sensitivity and 69% specificity [16]. Furthermore, Chapman *et al.* discriminated 10 MPM patients from 32 HC controls with 90% sensitivity and 91% specificity and from 18 ARD patients with 90% sensitivity and 83% specificity [17]. This may not be unexpected because of the lower number of patients and the fact we merged the sensor defections of 4 devices as final eNose profile.

Again, we could not discriminate ARD patients from AEx controls, which confirms our GC-MS findings. This could be due to the fact that 93% of the ARD patients had pleural plaques: the most common benign asbestos-related disease with collagen deposits in the mesothelium. Since there is no inflammatory aspect which can induce VOCs, no substantial VOC differences with AEx persons are to be expected. This allowed us to pool both groups which increased the discriminatory capacity.

The strength of our study lies in the multiple groups design and the replication of the results between two essentially different technologies for molecular assessment in exhaled air. Nevertheless, we acknowledge our study has important limitations. First of all, the low number of included participants restricts its application in the whole population. However, our results are in line with previous research and stresses its potential as screening tool. Secondly, our patients and controls were not matched for age and a trend in difference in

smoking status was found. This can be due to the long latency period between first asbestos-exposure and MPM diagnosis, delaying diagnosis to late stages in more elderly people. Furthermore, it is hard to find healthy controls without substantial comorbidities at matched age. The difference in smoking status can originate from the fact that asbestos workers were blue-collar workers; an industry known to have an increased incidence of smokers [29]. Nevertheless, since MPM development is independent of smoking, the impact of smoking status on our results is expected to be minimal and smoking-associated VOCs (benzene, 2,5-dimethylfuran, and toluene) were not selected in either model, underlining the independency. Thirdly, although an inspiratory VOC-filter was used, it is possible that exogenous compounds could have contaminated the breath since inhaled VOCs can be stored for a long time in the body's fat compartments [30], and the sampling and analysis materials used can also release compounds. Finally, despite a cross-sectional, case-control design, our study was not blinded and we took breath samples from participants with known diagnosis. The next step should be to perform a blinded, prospective, case-control, cohort study to assess the diagnostic features of the breath test.

Despite these limitations, we found MPM patients to be discriminated from the at risk groups with clinically relevant accuracy by both GC-MS and eNose analysis. The large sensitivity and NPV allows breath analysis to be used as screening tool for exclusion of disease in at risk persons and to enrich the fraction of individuals at risk for further screening. By doing so, not every asbestos-exposed person is subjected to repeated chest imaging procedures, which will help the monitoring of asbestos-exposed individuals to be more cost-effective and reduce the associated radiation exposure [31]. Future research should focus on the next step: validating our results in an independent, large, multicentre series with blinding of the investigator for the underlying disease, monitoring AEx persons over time and see how breath analysis can be used to screen for MPM. In addition, the VOCs should be linked with the pathophysiology of MPM by comparing the VOCs in breath with those in the headspace of mesothelioma cell lines and pleural fluid. This will ultimately improve the specificity.

CONCLUSION

GC-MS and eNose analysis allowed to discriminate MPM persons from asymptomatic, former asbestos-exposed persons at risk for MPM with great accuracy. The VOCs diethyl ether, methylcyclopentane, nonanal, limonene, cyclohexane, VOC I_K 1287 and isothiocyanatocyclohexane were identified as promising biomarkers for MPM. These data provide the proof-of-principle for future screening of persons at risk for MPM as a step-up tool in its diagnosis, making it less-invasive for the patient.

Declaration of interests:

None of the authors declare to have conflicts of interest. This study was presented at the ERS International Congress, 3-7 September 2016, London, UK.

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Supplementary Figures

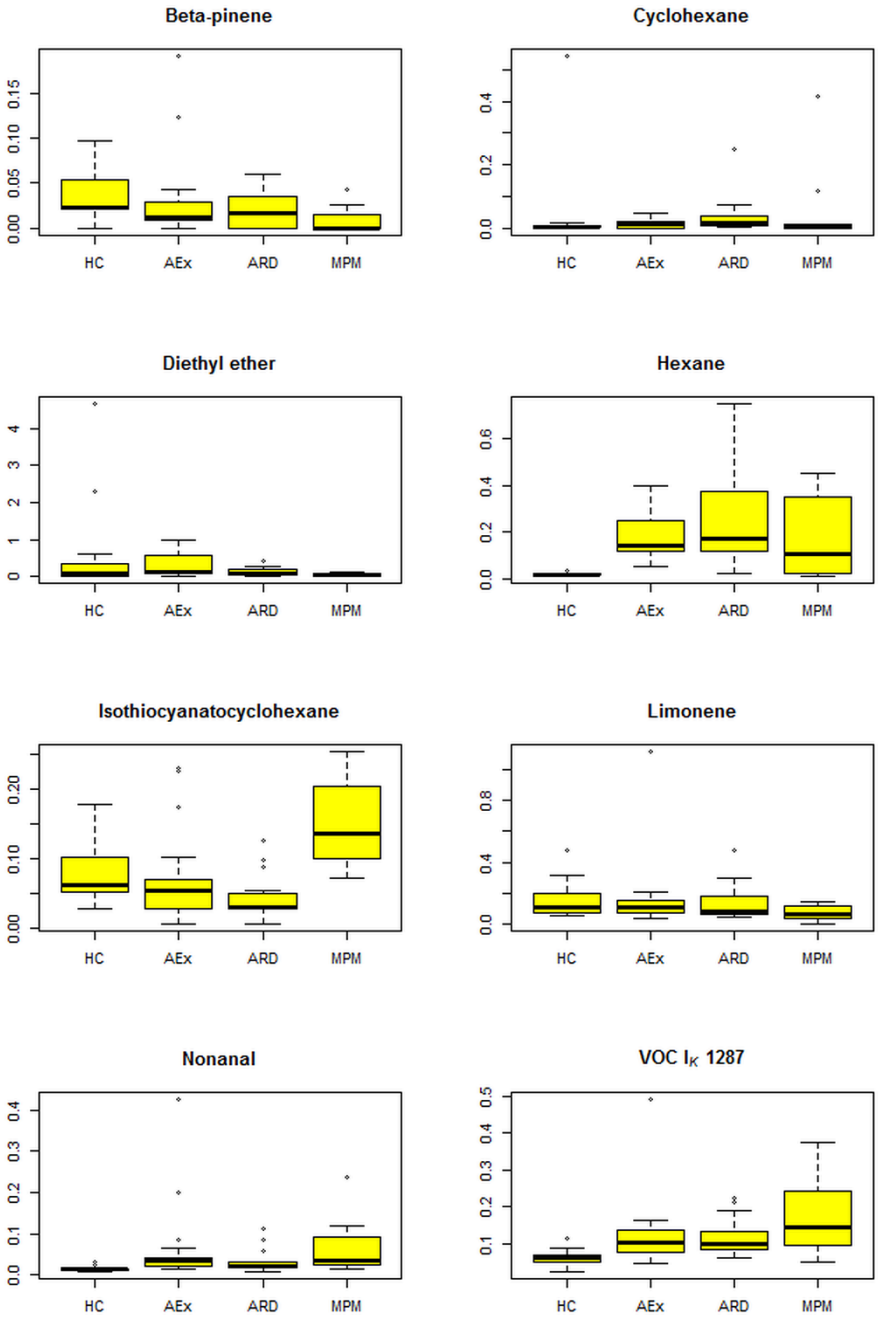


Figure S1: Boxplots of important selected VOCs by lasso regression. AEx: asymptomatic former asbestos-exposed controls. ARD: patients with benign asbestos related diseases. HC: healthy controls. I_K: Kováts retention index. MPM: malignant pleural mesothelioma. VOC: volatile organic compound.

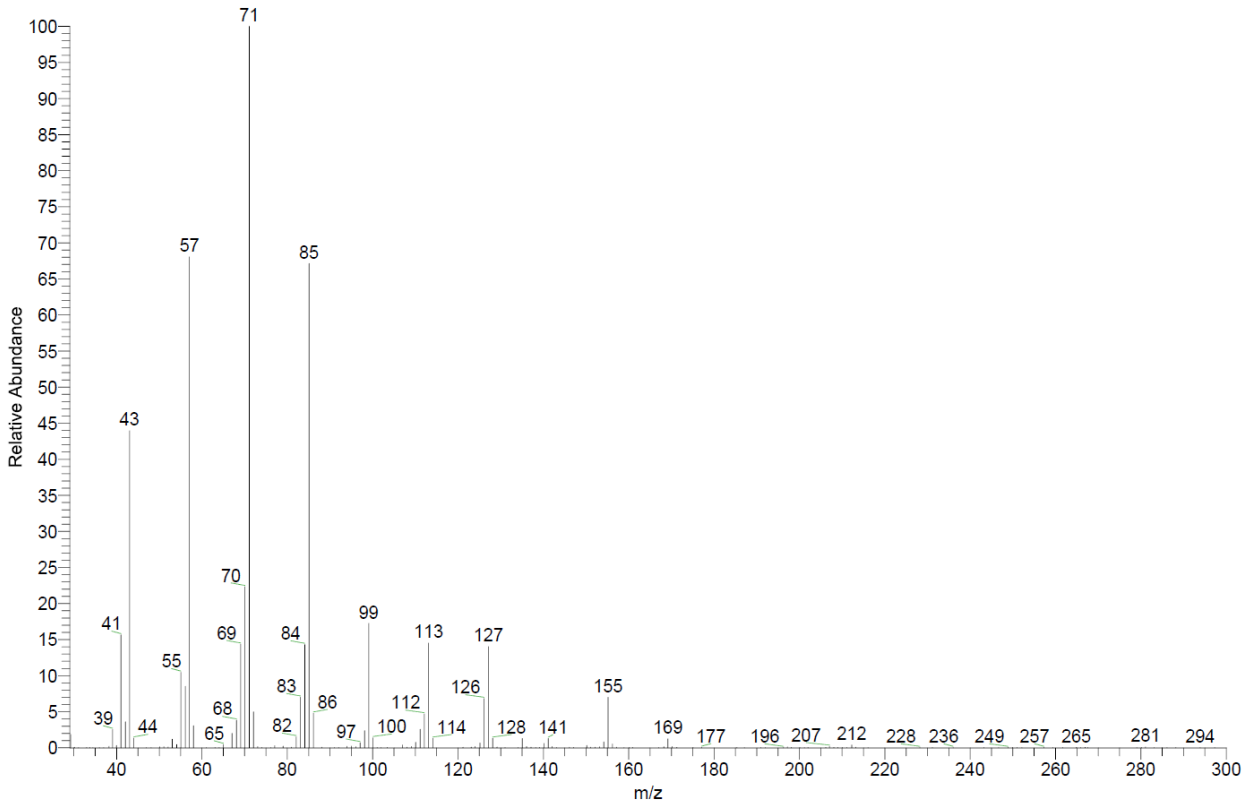
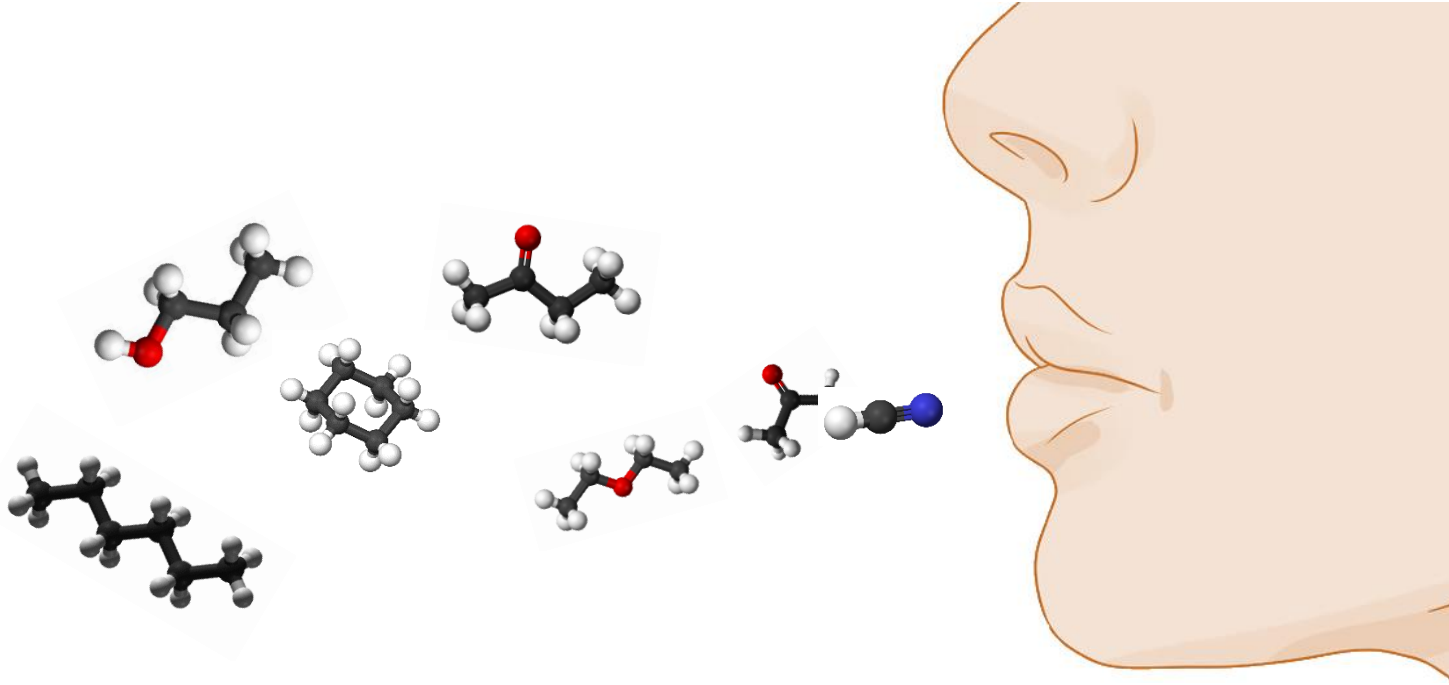
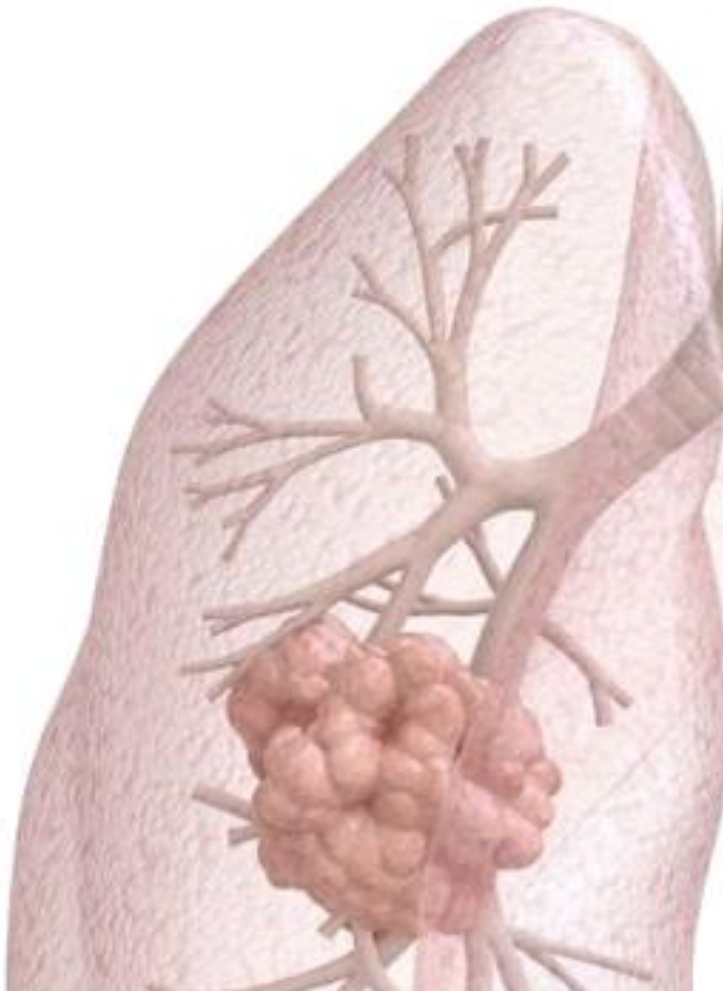


Figure S2: Mass spectrum of VOC I_K 1287.



PART IV:

GENERAL DISCUSSION & FUTURE PERSPECTIVES



CHAPTER 1
GENERAL DISCUSSION

An early detection of malignant pleural mesothelioma is hampered due to non-specific symptoms, which do not always manifest, and non-specific imaging techniques. MPM is formally diagnosed only after histopathological examination of a biopsy obtained through invasive thoracoscopy. Every asymptomatic person at risk with a suspected mesothelioma momentarily undergoes this procedure, leading to many false positives, increased morbidity from diagnostic delay or mistreatment, psychological stress, and unnecessary healthcare costs and procedures. Therefore, a screening tool which can help enrich the population at risk for further follow-up is urgently needed. It is essential such tools are accurate, associated with disease, and finally lead to an improved patient outcome by affecting the clinical decision making [1-5]. Important for diagnosis and screening is the accuracy of the diagnostic test which is determined by its sensitivity, NPV, specificity, and PPV [5, 6]. Considering the use for screening asbestos-exposed individuals at risk for mesothelioma, the breath test should have a high sensitivity and NPV to ensure that no patients will be missed and to rule out disease in the true negative population without the disease. Hence, maximizing sensitivity and NPV should be key for breathomics research regarding screening for mesothelioma. For diagnosing mesothelioma, the test should have a high specificity and PPV that allows to rule in diagnosis in the true positive patient population. However, when in this case a patient is false negative, the impact on the outcome is expected to be worse than when being false positive in a screening procedure, accompanying with an increased associated psychological stress and effects on quality of life.

Imaging screening studies have not been proven to be effective in identifying early mesothelioma in pleural tissue [7]. For blood biomarkers like SMRP, a meta-analysis showed that mesothelin hampers sensitivity to be used for the early diagnosis of mesothelioma [8], which is also seen in other independent studies [9]. However, SMRP could be useful as prognostic marker or for follow–up after mesothelioma diagnosis has been made [10, 11]. No single blood biomarker was found to be accurate enough for diagnosis of screening for mesothelioma. A biomarker panel combining two or more blood markers increases the potential, suggesting an important role for biomarker panels for screening and diagnosis [12-

14]. Since mesothelioma carcinogenesis is related to an increased oxidative stress [15, 16], we hypothesized that volatile organic compounds in breath could be of interest as biomarkers for screening. Therefore, this thesis aimed to elucidate the role of breath analysis for the screening and diagnosis of pleural mesothelioma and to initiate the first steps in biomarker development.

In our **first study**, we performed the first step in this process and aimed to explore the possibility to use breath analysis for mesothelioma diagnosis or screening and to identify discriminatory VOCs. We have demonstrated the feasibility of a standardised breath test using MCC/IMS and discriminated MPM patients from healthy asymptomatic persons with and without historical asbestos exposure with a clinically acceptable specificity and negative predictive value. Considering the rather low number of samples but high number of parameters, we opted to use a least absolute shrinkage and selection operator (lasso) regression method. Another aspect of why we chose to use this method is because of its ability to select a subset of important variables, which decreases the complexity of the final regression model and improves the interpretability, which is clinically useful [17]. From the inclusion of 89 VOCs in the lasso regression, we could identify the compounds P3, P5, P50, P71 and P87 as being the most important discriminators between patients and controls.

Our **second study** assessed the analytical validity of the test where we repeated the first study using the same protocol and analysing scheme. We initiated a cross-sectional, case-control study including more patients and controls and including groups that are important for the differential diagnosis of MPM and could confound the results, such as lung cancer patients and patients with benign asbestos-related and non-asbestos-related diseases. A total of 330 individuals were included (Part II, Table 4, page 85), which is believed to be the largest study assessing breath analysis for mesothelioma detection. In this study, MPM patients were discriminated from at risk groups with a large clinically relevant accuracy (82%-88%). The large sensitivity (87%-94%) and negative predictive value (83%-96%) allows breath analysis to be used as step-up screening tool in the diagnostic workflow for MPM. In this study, we analysed 250 VOCs instead of 89 in the first study. Again, we opted to use a lasso regression. The compounds selected by the lasso regression as the most discriminative were the VOCs P1, P3, P7, P9, P15, P21, and P26. It is interesting to see that P3 was retained from our previous proof-

of-principle study. Several factors can explain the modest discrepancies between the studies. First of all, a larger number of participants is included, increasing the power of the study to improve the differentiation of the groups. Furthermore, 250 VOCs were allowed to be selected in the model. This gives the opportunity to make clinical differentiations based upon a larger pool of possible variables, and explains the fact that some other compounds were selected as important discriminators. However, since P3 remained selected, this strongly suggests a possible role in MPM pathogenesis. Considering the discrimination between lung cancer and mesothelioma patients, only a modest discrimination was achieved (72% accuracy). Sensitivity (73%) and specificity (71%) were inadequate for clinical use, increasing the possibility that the discriminatory VOCs we found are rather compounds for general tumour detection instead of being specific for mesothelioma. Nevertheless, since we did obtain a modest discrimination, some of these VOCs must possess some kind of specificity for mesothelioma. Above of this, VOC P3 was not selected in the discrimination between MPM and lung cancer patients, stressing its potential use as MPM marker. Future research should focus on detecting these compounds (see Part IV, Chapter 2, page 163) and determine the clinical validity. One way to achieve this is by performing *in vitro* studies. The fact we obtained comparable accuracies compared to our previous study, confirms the analytical validity of breath analysis. Furthermore, we opted to perform a manual selection, optimization and analysis of the MCC/IMS VOC peaks, since it was shown to generate better results than automated peak selection and therefore is referred to as the gold standard [18]. However, since breath analysis yield huge amounts of data, future research should weigh the trade-off between a slightly higher accuracy when selecting peaks manually and a huge increase in processing speed when automated procedures are chosen.

The **final study** was performed in order to proof analytical validity and to identify VOCs important for the discrimination, since we only obtained a “pseudo-identification” from the MCC/IMS library. Therefore, we set up a cross-sectional, case-control study and used the gold standard (GC-MS) to differentiate MPM patients from the at risk control groups. Using GC-MS, mesothelioma patients were discriminated with 97% accuracy from persons previously exposed to asbestos, reaching a sensitivity and specificity of more than 90%. Even a combination with patients with benign asbestos-related diseases into a general group with asbestos-exposure improved the screening capabilities with a sensitivity and negative

predictive value of both 100%. The most important discriminatory VOCs were nonanal, diethyl ether, limonene, methylcyclopentane, cyclohexane, a VOC with Kováts retention index 1287, and isothiocyanatocyclohexane.

Despite the satisfying results from our studies, there are some critical points that need to be considered. First, for GC-MS analysis, only 113 VOCs were analysed from the plethora of VOCs that are already found in human breath, albeit not unequivocally identified [19, 20]. Second, because of the labour-intense effort in finding and optimizing the peaks, we selected only those compounds with a signal-to-noise ratio above 10 (S/N>10) and hence, are above the limit of quantification (LOQ). However, VOCs with a S/N between 3 and 10 (above the limit of detection; LOD), could also potentially be interesting to investigate. Subsequently, we cannot say with certainty that the VOCs we found with GC-MS are the ones found with MCC/IMS and vice versa. Therefore, a direct comparison between MCC/IMS and GC-MS samples is advocated in future research [21]. Furthermore, as with MCC/IMS measurements, it would be interesting to develop an automated peak finding algorithm that allows to select all optimized peaks with a S/N above the LOD in the GC-MS chromatogram [22]. This would lead to a faster and efficient peak selecting procedure which is less labour-intense and increases the number of potentially discriminatory VOCs. However, one has to cope with possible co-eluting compounds and has to select and integrate the compounds in an equal way. Lastly, for some of our VOCs found with GC-MS, we do not have a definite identification after cross-checking with the mass spectral library of the National Institute of Standards and Technology (NIST). These VOCs are reported by their Kováts retention index, which allows pseudo-identification, and further steps are needed to allow their final identification. VOC identification is also tentative since we cannot exclude that the VOCs we identified as important are co-eluting compounds if they have the same mass-to-charge (m/z) ratio and spectral pattern (for instance, because these are isomers) [23]. To exclude potential co-eluting compounds, the use of special software [24], or extra measurements with GCxGC-MS (2D-GC-MS) or separations even up to four dimensions can solve this issue [25-27]. However, this will be accompanied with an increased research cost.

The strength of our study lies also in the fact that we took parallel breath samples from some patients to validate our GC-MS findings with sensor technology, which are essentially different technologies for molecular assessment in exhaled air. This was the analytical validity of the test and generated in-line results, albeit with lower discriminatory characteristics. This is due

to the lower number of samples and the fact that electronic noses generate a breathprint using the bulk of the breath instead of looking at specific compounds. This underlines the fact that by being more specific and focussing on specific compounds (as with GC-MS), you can increase the discriminatory capacity. As with SMRP in blood, no single VOC biomarker was selected as being important and all studies had an acceptable discrimination due to the combination of VOCs. This stresses the importance of biomarker panels instead of single markers for disease detection and screening, certainly in a heterogeneous disease like MPM.

In general, there are some characteristics that we need to account for when dealing with breath analysis. Despite our studies are being matched for gender, and the gender balance is comparable to what is reported for mesothelioma, and several studies found gender not to influence VOCs [28-31], others did find an effect of gender on the VOC levels [32, 33]. Therefore, we cannot fully exclude gender-specific variations. Next to this, we were not able to match for age since it is hard to find matched healthy controls without comorbidities at that age. Several studies have shown some VOCs to be associated with increasing age [33, 34], while others did not find any association between VOCs and ageing [31, 35-37]. Therefore, we cannot fully exclude the possibility that certain VOCs are linked with this age difference. Furthermore, given the fact that physical exercise increases the cardiac output and metabolism, we can expect differences in VOC depending on the exercise level. Several studies focused on the effect of exercise on the VOC composition of breath, especially isoprene and acetone [38-42], or showed that exercise could change the breathprint of healthy subjects [43]. Next to this, it has been shown that the expiratory flow rate, breath holding manoeuvres or including the dead space in the analysis induced significant changes in the breathprints of healthy volunteers [44, 45]. Therefore, we opted to sample alveolar air, with the patient being in a relaxed state, sitting upright after a resting period and breathing normally without performing any forced breathing manoeuvres. Furthermore, a numerous amount of compounds can be derived from inspired air or from food intake [46-48]. For inspired air, several studies used inspiratory VOC filters to exclude environmental VOCs. However, this is not straightforward because exogenous compounds can be stored in the body tissues for a long time [49, 50] or can undergo metabolic changes before exhalation [51]. Next, there is no consensus on how to deal with background contamination. We decided to take a background sample for every participant and chose to calculate the alveolar gradient of the compounds.

By doing so, we try not to be stringent by ignoring every VOC that is present in the environment in the breath sample and try to account for VOCs that are stored in the body. Furthermore, this allowed us to further reduce the noise by compensating for contamination coming from the used sampling materials. As mentioned, the diet could also induce a change in VOCs [52-55]. After eating, the food is being metabolized and could ameliorate VOC changes. After an inspection of the literature, we used a fasting period of 2 hours, which was applied the most in breath research. However, it still needs to be elucidated if this time frame is large enough.

Another important source of exhaled VOCs are the gut microbiome or respiratory microbial infections [56-59]. These VOCs could help to identify respiratory infections but their effect in mesothelioma is expected to be minimal. However, these compounds could potentially help in differentiating pneumonia from other diseases. Considering all of the above-mentioned pitfalls in breath analysis, the International Association on Breath Research (IABR) founded an international consortium to address the issues concerning the sampling, storage and analysis of breath samples [60, 61], and progress in these different aspects of breath analysis can be expected in the upcoming years.

In conclusion, we can say that with MCC/IMS, we were able to discriminate MPM patients from the population at risk for mesothelioma with a clinically high sensitivity and negative predictive value (Part III, Chapters 1 and 2), making it optimal to rule out disease in those asymptomatic persons at risk and enrich the population for further screening methods or biopsy, for instance with CT and thoracoscopy. This was validated with GC-MS (Part III, Chapter 3). Considering MPM versus asymptomatic persons exposed to asbestos fibres, we obtained 93% sensitivity and 95% NPV. Above of this, the specificity and PPV were 100%, also allowing the tool to be used for diagnostic purposes. With an eNose, we generated in-line results but the low specificity suggests to use the breath test as screening tool rather than for diagnosing MPM. Future research should focus on these aspects and continue the pipeline in biomarker development. This is discussed in the next chapter.

CHAPTER 2
FUTURE PERSPECTIVES

This thesis comprises the first steps in biomarker development: the discovery of biomarkers for MPM screening and determination of the analytical validity of breath sampling (Part I, Chapter 6). However, several steps have to be taken before a biomarker can be implemented into the clinic. The next step in biomarker discovery is to find biological evidence for the presence of the VOCs in breath. Therefore, we have initiated a third phase of the MesoBreath series in which we will analyse the headspace of different mesothelioma and lung cancer cell lines. This *in vitro* study is necessary as it will form the translational bridge between the breath test and the tumour's metabolism or associated inflammation. This will lead to the identification of key compounds that are related to the tumour's metabolism and assess the clinical validity of breath analysis for MPM.

However, in our *in vivo* research, MPM patients had only a moderate separation from lung cancer patients, probably due to a common tumour metabolism. By looking which compounds differ between MPM and lung cancer cell lines, we can focus only on these and by confirming their presence in breath, it will ultimately improve the specificity of the breath test [62]. Above of this, the *in vitro* cell lines could potentially be subjected to hypoxic conditions that imitates the *in vivo* situation. Furthermore, the next step also includes to explore a xenograft mesothelioma mouse model since this translational model mimics the tumour in its natural environment. By sampling xenograft mice with mesothelioma, VOCs in early stage mesothelioma could potentially be identified.

When several compounds are selected as being key in discriminating MPM from the at risk population, specific sensors for these compounds could be built and combined into a hand-held eNose specifically for MPM. This than should again be tested in the population to assess its clinical utility. In order to do so, a large, prospective, case-control cohort study should be initiated that allows to follow an at risk population over time. By taking breath samples over time at certain time intervals, VOCs can be followed and their kinetics studied. This test should also be compared with the current diagnostic work-up, resulting in a direct comparison with a CT scan and biopsy. Furthermore, since our research did not suggest a role of breath analysis for diagnosing, but rather for screening, the place of breath analysis in the mesothelioma

diagnostic workflow has to be determined. The prospective study should assess the clinical utility of breath analysis before any other diagnostic work-up and see if it allows us to enrich the patients at risk for further screening. When the test result is negative for a specific test person, he or she should not be considered for further screening. On the other hand, it could also be used after CT-scan when there is doubt about the presence of a tumour. In that way, only patients positive for the breath test could be referred for biopsy or patients with a negative test can be excluded for further biopsy.

Another element to investigate is the combination of biomarkers into biomarker panels. In the prospective study, blood samples could be taken to measure the SMRP levels in parallel to a breath sample. Combining breath VOCs with SMRP could complement each other and increase the detection characteristics. In the end, a high sensitivity and negative predictive value for the test would allow to rule out disease in the true negative population, thereby excluding these from an invasive diagnostic course. This will ultimately help the diagnosis for MPM to be more efficient which results in a more cost-effective screening and decrease the burden of radiation for the patient.

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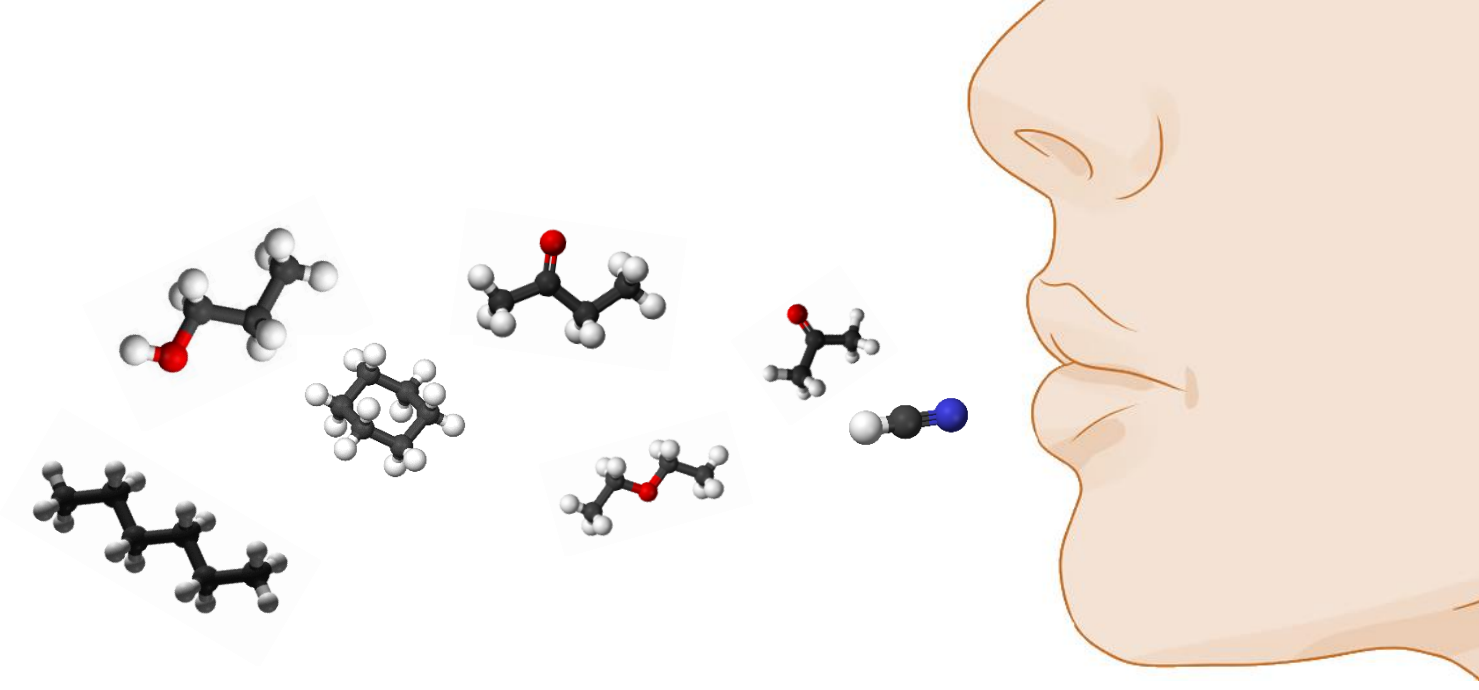

SUMMARY
SAMENVATTING

SUMMARY

Malignant pleural mesothelioma (MPM) is an aggressive malignancy originating from the layer that covers the thoracic cavity and the lung and is causally associated with previous asbestos exposure. Despite a ban on asbestos use in the entire European Union in 2005, asbestos is still being produced and consumed in several countries in need for industrial growth. Together with a long average latency period of 40 to 50 years between first asbestos exposure and MPM diagnosis, this indicates that MPM incidence will further increase. The mean age at diagnosis is 69 years. There are worldwide differences in incidence ranging from 40 per million inhabitants in Australia to 9 per million in the US. Europe has in incidence rate of 20 per million inhabitants with large intercountry differences. For Belgium, the cancer registry estimated an incidence rate of 39 per million male inhabitants in 2008, one of the highest worldwide due to the large asbestos consumption in the past. With a 5-year survival rate below 5%, prognosis remains poor, stressing the need for an earlier diagnosis by screening. Clinical symptoms (dyspnoea, chest pain) and imaging techniques are not specific enough and are therefore not advocated as screening tools. Serum biomarkers, like serum mesothelin-related peptide (SMRP), were not optimal for use as a stand-alone diagnostic marker with sensitivities ranging from 19% to 68% and specificities ranging from 88% to 100%, Presently, any screening for mesothelioma in asbestos-exposed individuals is considered futile and hence not recommended. MPM is only diagnosed after examination of a biopsy obtained after thoracoscopy.

The quest for simple, non-invasive diagnostic tests in respiratory medicine has recently shifted to the analysis of exhaled breath. Breath contains volatile organic compounds (VOCs) in pico-molar concentrations which arise from the body's (patho)physiological processes, enter the bloodstream and enter the lung alveoli by gas exchange mechanisms. VOCs have already demonstrated to be useful in the detection of asthma, COPD, and several tumours like breast cancer. There are over 3000 different VOCs detected in human breath. Since the first identification of VOCs in 1971, the field of breath analysis evolved into a high-throughput *breathomics* field, focusing on sampling and statistical procedures to translate breath analysis to the clinic.

This research work focusses on the role of breath analysis for mesothelioma diagnosis and screening. Before this study was initiated, several studies provided the proof-of-principle wherein the breath of groups with small sample sizes was analysed and wherein MPM patients could be separated from controls using gas chromatography – mass spectrometry (GC-MS) and pattern recognition tools as electronic noses (eNoses). However, we wanted to take the next step in biomarker development and use multicapillary column-ion mobility spectrometry (MCC-IMS) as breath analysing tool because it allows a (pseudo)identification of VOCs, can be used at the patients' bedside or in the spirometry laboratory and is less costly than GC-MS, the gold standard. In order to obtain a large and relevant patient population, we set up a Belgian multicentre, case-control study. This allowed us to evaluate breath analysis in one of the largest studies concerning breath analysis for MPM. We included 52 mesothelioma patients and 278 control subjects consisting of healthy controls with and without historical asbestos exposure, patients with benign asbestos-related diseases, patients with benign respiratory diseases unrelated to asbestos exposure and lung cancer patients.

We first looked whether MCC/IMS was able to discriminate MPM patients from healthy controls with and without historical occupational asbestos exposure. The diagnostic accuracies were satisfying and several VOCs were selected as being important discriminators, allowing us to proceed our research.

The next step was to see if our results could be replicated in larger samples and by including other control groups. This gives us the analytical validity of the test and ultimately helps identifying important VOCs. The results of this study confirm our previous findings. We discriminated mesothelioma patients from asymptomatic persons with historical asbestos exposure with 88% accuracy. When adding patients with benign asbestos-related diseases to the at risk control population, the sensitivity and specificity of the breath test were 94% and 96%, respectively, indicating the use of breath analysis as screening tool. Nevertheless, MPM patients were only moderately discriminated from lung cancer patients (77% accuracy), suggesting a more common tumour metabolism. This study provided the analytical validity of the MCC/IMS breath test.

Since we only obtained a pseudo-identification of the VOCs, we performed another cross-sectional, case-control study in which we analysed the breath of a subset of patients with two different technologies for the molecular assessment of breath. By using GC-MS, the gold standard in breath analysis, we discriminated MPM patients from both control groups with

known asbestos exposure with 94% accuracy and 100% sensitivity and negative predictive value. We have identified the compounds diethyl ether, methylcyclopentane, nonanal, limonene, cyclohexane, isothiocyanatocyclohexane and a VOC with Kováts retention index 1287 as important discriminators. Above-of-this, we took parallel samples in these patients for eNose analysis to validate the findings. This generated in-line results, underlining the potential to use handheld sensor technology for future screening of persons exposed to asbestos.

In conclusion, we have shown that breath analysis by MCC/IMS was able to discriminate MPM patients from subjects at risk and its analytical validity was assessed. Furthermore, by GC-MS analysis and eNose validation, we identified important discriminators and showed the potential use of sensors for screening. We found that the use of breath analysis for early MPM diagnosis is limited due to the lower specificity of the tests. However, its potential for screening has been identified considering the large sensitivity and negative predictive value. This allows to rule out disease in true negative subjects and enrich the population at risk for further screening with conventional methods. This should allow screening to be more cost-effective, limiting unnecessary procedures and associated radiation exposure in all persons at risk. Future research should address this in a prospective, multicentre, cohort study and following subjects with past asbestos exposure over time.

SAMENVATTING

Kwaadaardig pleuraal mesothelioom, ook wel longvlieskanker genoemd, is een zeer agressieve tumor dat ontstaat uit het mesotheel, een vlies dat de binnenkant van de borstholte bedekt en zich rond de longen bevindt. Het ontstaat na blootstelling aan asbestvezels. Dit type kanker wordt gekenmerkt door een lange latentieperiode tussen de blootstelling aan asbestvezels en de diagnose van gemiddeld 40 à 50 jaar. In de westerse landen situeert de gemiddelde leeftijd bij diagnose zich dan ook rond 69 jaar. Desondanks een verbod op het gebruik van asbest in meer dan 55 landen waaronder alle landen van de Europese Unie, wordt het toch nog gewonnen en verwerkt in gebieden met laag inkomen waar de nood aan industriële groei het grootst is. Dit zorgt er voor dat het aantal gevallen met longvlieskanker nog zal stijgen in de toekomst. Er zijn wereldwijd duidelijke verschillen in de gerapporteerde incidentie van mesothelioom. In Australië worden er jaarlijks 40 per miljoen inwoners getroffen door mesothelioom terwijl dit in de Verenigde Staten 9 per miljoen is. In Europa ligt de incidentie rond 20 per miljoen inwoners met grote verschillen tussen de landen. Het Belgisch kankerregister meldt voor 2008 een incidentieratio van 39 per miljoen mannelijke inwoners, één van de hoogste wereldwijd. Dit is een gevolg van ons hoge asbestgebruik in het verleden.

Longvlieskanker heeft een slechte prognose: de mediane overleving van onbehandelde patiënten is 6-9 maand en minder dan 5% van de patiënten is nog in leven na 5 jaar. De klinische symptomen van mesothelioom (kortademigheid, pijn in de borstkas, hoest) zijn meestal niet-specifiek en bedrieglijk. Ze kunnen dan ook niet gebruikt worden als enige diagnostische criterium, ook niet in het geval van voorafgaande asbestblootstelling. Beeldvorming kan suggestief zijn voor mesothelioom, maar kan ook niet aangewend worden voor de eigenlijke diagnose. Om met zekerheid de diagnose van longvlieskanker te stellen is een thoracoscopie aangewezen waarbij weefsel wordt weggehaald voor analyse. De aspecifieke en laattijdig zichtbare ziekteverschijnselen alsook de diagnostische moeilijkheden belemmeren een vroegtijdige diagnose en bijgevolg ook een mogelijks efficiënte behandeling. Vorig onderzoek naar diagnostische biomerkers in het bloed, zoals het serum mesotheline-gerelateerd peptide (SMRP), heeft aangetoond dat deze op zichzelf niet bruikbaar is voor de diagnose van longvlieskanker door een te lage gevoeligheid (19-68%). Om diezelfde reden is

screening voor longvlieskanker in asbest-blootgestelde personen daarom ook niet aangewezen.

De zoektocht naar een eenvoudige, niet-invasieve diagnostische biomerker leidde recent naar het veld van de ademanalyse. De menselijke adem bevat een groot aantal vluchtige organische componenten (VOCs) in pico-molaire concentraties die kunnen dienen als diagnostische merkers. VOCs komen voort uit endogene biochemische processen die gerelateerd kunnen zijn aan ziekte-specifieke mechanismen. Deze worden vrijgesteld in het bloed en naar de longen vervoerd waarna ze door de gas uitwisselingsmechanismen in de adem terechtkomen. Dit vernieuwend type biomerker heeft reeds zijn rol bewezen in verschillende pulmonale aandoeningen, zoals astma, chronisch obstructief longlijden en andere tumoren zoals borstkanker.

Met de voorliggende studie willen we een ademtest ontwikkelen en valideren voor vroegtijdige diagnose van mesothelioom. Voorafgaand aan dit onderzoek hebben verschillende kleinschalige studies aangetoond dat ademanalyse wel kan werken, waarbij patiënten met longvlieskanker konden worden onderscheiden van een risicopopulatie met gas chromatografie – massa spectrometrie (GC-MS) en het gebruik van patroonherkenning zoals een elektronische neus (eNose). Met dit onderzoek willen we een stap verder gaan in de ontwikkeling van een ademtest voor longvlieskanker en nagaan of multi-capillaire kolom/ion mobiliteit spectrometrie (MCC/IMS) eveneens bruikbaar is als analysemethode omwille van het feit dat deze een (pseudo)identificatie toelaat van de VOCs, gemakkelijk te transporteren is naar de patiënt en goedkoper is dan GC-MS analyse, de gouden standaard. Om een zo groot mogelijke patiëntenpopulatie te verkrijgen hebben we een multicenter, patiënt-controle studie opgezet. Dit laat ons toe om ademanalyse voor de diagnose en screening van mesothelioom te evalueren in een van de grootste studies in het veld van de ademanalyse. We hebben 52 mesothelioompatiënten en 278 controle personen geïncludeerd bestaande uit gezonde personen met en zonder voorafgaande asbestblootstelling, patiënten met goedaardige asbest-gerelateerde en niet asbest-gerelateerde aandoeningen en longkankerpatiënten.

In een eerste fase hebben we nagegaan of MCC/IMS bruikbaar is om mesothelioompatiënten te onderscheiden van controlepersonen met verhoogd risico. De accuraatheid van de discriminatie was tevredenstellend en bepaalde VOCs werden aangeduid als zijnde belangrijk in dit onderscheid.

Hierna hebben we nagegaan of we dezelfde resultaten verkregen als we de studie herhalen in een groter aantal patiënten en als we groepen includeren die een differentiaaldiagnose bemoeilijken. Dit geeft ons de analytische validiteit van de test en zal ons helpen om bepaalde VOCs aan te duiden als belangrijke discriminatoren. Deze studie bevestigde ons voorgaand onderzoek: we konden mesothelioompatiënten onderscheiden van asymptomatische personen met voorafgaande asbestblootstelling met 88% accuraatheid. Wanneer personen met een goedaardige asbest-gerelateerde aandoening aan de controlegroep werden toegevoegd waren de gevoeligheid en specificiteit respectievelijk 94% en 96%. Dit benadrukt het gebruik van ademanalyse voor het screenen van mesothelioom in een risicopopulatie. Mesothelioompatiënten konden echter maar matig worden onderscheiden van longkanker patiënten (77% accuraatheid), wat op een gemeenschappelijk tumormetabolisme kan wijzen. Gezien we tot nu toe enkel een “pseudo-identificatie” van de VOCs verkregen, hebben we finaal een nieuwe cross-sectionele, patiënt-controle studie opgezet. De opzet was om adem te analyseren met twee totaal verschillende types technologie dat toelaat om de moleculaire samenstelling van de adem te onderzoeken, met name GC-MS en eNose. GC-MS, de gouden standaard in ademonderzoek, liet ons toe om mesothelioompatiënten te onderscheiden van beide controlegroepen met gekende asbestblootstelling met 94% accuraatheid en 100% gevoeligheid en negatief voorspellende waarde. We hebben de componenten diethyl ether, methylcyclopentaan, nonanal, limoneen, cyclohexaan, isothiocyanatocyclohexaan en een VOC met een Kováts retentie index 1287 geïdentificeerd als zijnde belangrijk. Hiernaast hebben we ademstalen in parallel afgenomen voor analyse met een eNose. Dit resulteerde in vergelijkbare resultaten en benadrukt de mogelijkheid om kleine sensoren te gebruiken in een toekomstig screeningsprogramma.

Samengevat hebben we aangetoond dat ademanalyse met behulp van MCC/IMS toelaat om mesothelioompatiënten te onderscheiden van personen met een risico op de tumor en hebben we de analytische validiteit van de methode aangetoond. Vervolgens hebben we de resultaten gevalideerd met GC-MS en eNose analyses en hebben we belangrijke discriminatoren voor het onderscheid tussen deze groepen aangeduid als mogelijke adem biomerkers. We vonden dat het gebruik van ademanalyse voor de diagnose van mesothelioom eerder gelimiteerd is door een lagere specificiteit, maar dat het potentieel voor screening is bewezen gezien een grote gevoeligheid en negatief voorspellende waarde. Dit laat toe om de

ziekte uit te sluiten in de echte personen zonder ziekte en op die manier de risicopopulatie aan te rijken voor verder onderzoek met de conventionele methoden. Dit zou moeten toelaten om op een meer kosteneffectieve manier te screenen, waarbij risicopersonen geen onnodige onderzoeken krijgen en een mindere blootstelling aan de straling afkomstig van de beeldvormingstechnieken. Verder onderzoek moet dit nagaan in een prospectieve, multicenter, cohort studie waarbij personen met een gekende asbestblootstelling worden gevolgd over de tijd.

CURRICULUM VITAE

PERSONAL INFORMATION

Name:	Kevin LAMOTE
Date of Birth:	June 3, 1988
Nationality:	Belgian
Office address:	University Hospital Ghent – 7K12IE
	De Pintelaan 185
	9000 Ghent, Belgium
Tel:	+32 (0)9 332 45 98
Fax:	+32 (0)9 332 23 41
e-mail:	kevin_lamote@hotmail.com
websites:	https://www.researchgate.net/profile/Kevin_Lamote
	https://be/linkedin.com/in/kevin-lamote-80a57156
	orcid.org/0000-0002-9500-7461

TRAINING AND CERTIFICATES

- *2009:* Bachelor of Science in Biomedical Sciences, cum laude
- *2011:* Master of Science in Biomedical Sciences, magna cum laude
- Course on 'Good Clinical Practice' *(February 2012, Ghent, Belgium)*
- Clinical Studies *(August 2012, Ghent, Belgium)*
- Statistical Beginners Course SPSS *(September 2012, Ghent, Belgium)*
- Meeting Skills *(September 2012, Ghent, Belgium)*
- Statistical Course R *(October 2012, Ghent, Belgium)*
- Personal Effectiveness *(October-November 2012, Ghent, Belgium)*
- Communication Skills Basic Course *(November 2012, Ghent, Belgium)*
- Data Mining *(January 2013, Ghent, Belgium)*
- Applied Linear Regression *(February-March 2013, Ghent, Belgium)*
- ERS Course on Breath Monitoring in Asthma, COPD and Pulmonary Diseases *(March 2013, CHU de Liège, Liège)*
- Advanced Academic English Conference skills *(February-May 2014, Ghent, Belgium)*
- Statistical Expert Course SPSS *(January-February 2015, Ghent, Belgium)*
- Master Class on Archival Breath Sampling (*April 2016, Loughborough, UK*)

EXPERIENCE

- 2011-2016: PhD candidate in Health Sciences, Faculty of Life Sciences and Medicine, Ghent University: *Volatile organic compounds and malignant pleural mesothelioma: simply breathtaking?*
- 2009-2011: Master thesis at the lab for Biomaterials (Ghent University Hospital): *the effect of mesoporous silica on the physical and osteogenic effects of calcium phosphate cements.*
- 2010: Laboratory Animal Science Accreditation FELASA level C.
- 2007-2009: Teaching assistant at the laboratory of Histology, Ghent University Hospital, Ghent, Belgium.

CONGRESSES AND SYMPOSIA

- EORTC-NCI-ASCO Annual Meeting on "Molecular Markers in Cancer" *(October 2011, Brussels, Belgium)*

- LONG Symposium: Thoracic Oncology: Small samples, big issues *(March 2012, Ghent, Belgium)*

- Science Day *(March 2012, Ghent, Belgium)*
  Poster presentation: The MesoBreath study: exhaled breath as diagnostic tool for malignant pleural mesothelioma – results of a pilot study.

- BVP/SPB - GSK Awards in Pneumology *(April 2012, Brussels, Belgium)*
  Oral presentation: Exhaled breath as diagnostic tool for malignant pleural mesothelioma: a pilot phase.

- BVP/SPB - GSK Awards in Pneumology *(Oral presentation, March 2013, Brussels, Belgium)*
  Oral presentation: Does haptoglobin play a role in malignant pleural mesothelioma?

- Science Day *(March 2013, Ghent, Belgium)*

- Breath Summit 2013 *(June, Saarbrücken/Wallerfangen, Germany)*
  Poster presentation: Volatile organic compounds in the breath of cystic fibrosis patients: a pilot phase.

- IASLC World Conference on Lung Cancer 2013 (*October 2013, Sydney, Australia*)
  Poster presentation: Volatile organic compounds as diagnostic tool for malignant pleural mesothelioma.

- EORTC-NCI-ASCO Annual Meeting on "Molecular Markers in Cancer" (*November 2013, Brussels, Belgium*)
  Poster presentation: Volatile Organic compounds as a diagnostic tool for malignant pleural mesothelioma.

- OncoPoint Meeting (*February 2014, Ghent, Belgium*)
  Oral presentation: Volatile Organic compounds as a diagnostic tool for malignant pleural mesothelioma.

- BVP/SPB - GSK Awards in Pneumology *(June 2014, Brussels, Belgium)*
  Oral presentation: Exhaled breath as diagnostic tool for malignant pleural mesothelioma.

- Breath Summit 2014 (*July 2014, Torún, Poland*)
  Poster presentation: Breath analysis for the diagnosis of malignant pleural mesothelioma.

- European Respiratory Society Congress 2014 (*September 2014, Munich, Germany*)
  Oral presentation: exhaled breath as a diagnostic tool for malignant pleural mesothelioma.

- ESMO 2014 Congress (September 2014, Madrid, Spain)
  Poster Presentation: A breath test for diagnosing malignant pleural mesothelioma.

- 11th Thoracic Oncology Winter Symposium: A flair of the future (*January 2015, Antwerp, Belgium*)
  Oral presentation: Volatomics for early diagnosis of lung cancer and mesothelioma.

- AACR Annual Meeting 2015 (*April 2015, Philadelphia, US*)
  Poster presentation: Exhaled breath for the diagnosis of malignant pleural mesothelioma.

- BVP/SPB - GSK Awards in Pneumology *(May 2015, Brussels, Belgium)*
  Oral presentation: Volatile organic compounds increase the likelihood of detecting malignant pleural mesothelioma.

- IASLC World Conference on Lung Cancer 2015 (*September 2015, Denver, US*)
  Mini Oral presentation: Breath analysis for the diagnosis of malignant pleural mesothelioma.

- European Respiratory Society Congress 2015 (*September 2015, Amsterdam, The Netherlands*)
  Oral presentation: Volatile organic compounds increase the likelihood of detecting malignant pleural mesothelioma.

Poster discussant for occupational and environmental exposure assessment and biomarkers.

- 12th Thoracic Oncology Winter Symposium (*January 2016, Ghent, Belgium*)

- Master Class on Archival Breath Sampling (*April 2016, Loughborough, UK*)
  Poster presentation: Volatile organic compounds increase the likelihood of detecting malignant pleural mesothelioma.

- International Mesothelioma interest Group (iMig) Meeting 2016 (*May 1-4, 2016, Birmingham, UK)*
  Oral presentation: Haptoglobin phenotype is a risk factor for malignant pleural mesothelioma.

- BVP/SPB - GSK Awards in Pneumology *(May 2016, Brussels, Belgium)*
  Oral presentation: A breath test for malignant pleural mesothelioma: a validation study.

- European Respiratory Society Congress 2016 (*September 2016, London, United Kingdom*)
  Oral presentation: Breath analysis by gas chromatography-mass spectrometry and eNose can be used to screen for malignant pleural mesothelioma.
  Poster Discussion: Analysis of volatile organic compounds in headspace air of mesothelioma cancer cell lines: experimental design

- Belgian Pneumology Days *(December 2016, Antwerp, Belgium)*
  Oral presentation: A breath test for malignant pleural mesothelioma: a validation study.

- IASLC World Conference on Lung Cancer 2016 (*December 2016, Vienna, Austria*)
  Oral presentation: Breath analysis by gas chromatography-mass spectrometry can be used to screen for malignant pleural mesothelioma.

MEMBERSHIPS

2011 - 2016: Belgian Society for Pneumology (BVP/SBP)
2012 - 2016: European Respiratory Society (ERS)
2012 - 2016: International Association for Breath Research (IABR)
2015 - 2016: International Association for the Study of Lung Cancer (IASLC)
2015 - 2016: American Association for Cancer Research (AACR)

GRANTS AND AWARDS

2016: GSK Clinical Science Award
2016: iMig Young Investigator Award
2015: Emmanuel van der Schueren Grant *(Flemish League Against Cancer)*
2015: ERS Young Scientist Sponsorship
2013: Foundation Against Cancer Grant STK 2012-223

PUBLICATIONS

Articles (A1)

1. Lamote K, Delange S, De Smet R, van Meerbeeck JP, Delanghe JR. Haptoglobin phenotype: a heritable factor for malignant pleural mesothelioma? A case-control study. *[Manuscript in preparation]*
2. Lamote K, Vynck M, Thas O, Van Cleemput J, Nackaerts K, van Meerbeeck JP. Ion mobility spectrometry for screening for malignant pleural mesothelioma: a validation study. *European Respiratory Journal. 2017. [Submitted]*
3. Lagniau S, Lamote K, Vermaelen KY, van Meerbeeck JP. Early diagnosis of malignant mesothelioma by biomarker: do we need another moonshot? Oncotarget. 2017 *[In Revision]*
4. Lamote K, Brinkman P, Vandermeersch L, Vynck M, Sterk PJ, Van Langenhove H, Thas O, Van Cleemput J, Nackaerts K, van Meerbeeck JP. Validation of breath analysis as screening tool for malignant pleural mesothelioma: a cross-sectional, case-control study. *Oncotarget.* 2017. *[In Revision]*
5. Lamote K, Vynck M, Van Cleemput J, Thas O, Nackaerts K, van Meerbeeck JP. Detection of malignant pleural mesothelioma in exhaled breath by multicapillary column/ion mobility spectrometry (MCC/IMS). *Journal of Breath Research*. 2016:10(4):046001. **[IF: 4.177; ranking: 11/77 (Q1; D2)]**
6. Lamote K, van Meerbeeck JP. Breathomics: niet-invasieve ademanalyse voor longkanker diagnose. OncoHemato. 2015;6:19-28. *[Article in Dutch]*
7. Lamote K, van Meerbeeck JP. Breathomics: analyse respiratoire non invasive pour le diagnostic du cancer pulmonaire. OncoHemato. 2015;6:19-28. *[Article in French]*
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Conference proceedings (P1)

1. Lamote K, Vandermeersch L, Van Langenhove H, Van Cleemput J, Nackaerts K, van Meerbeeck JP. OA22.05 Breath analysis by gas chromatography-mass spectrometry can be used to screen for pleural mesothelioma. Journal of Thoracic Oncology. 2017;12(1):S331.

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3. Lagniau S, Lamote K, Vermaelen K, van Meerbeeck JP. VOC analysis in headspace air of mesothelioma and lung cancer cell lines: A comparative literature study. European Respiratory Journal. 2016;48(60):PA5080.

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10. van Meerbeeck JP, Lamote K. Screening Tools for a High Risk Population-Can We Screen for Early Mesothelioma? Journal of Thoracic Oncology. 2013;8:S107-S108.
11. Lamote K, Van Cleemput J, Nackaerts K, Van Meerbeeck JP. Volatile Organic Compounds as Diagnostic Tool for Malignant Pleural Mesothelioma. Journal of Thoracic Oncology. 2013;8:S1234-S1234.
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